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Refinement and validation of a PCR test to replace WEC and FCLD, including commercial feasibility

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Abstract

The objective measurement of disease organisms in the environment and in animals will help livestock owners manage disease. Current methods for assessing gastrointestinal nematode parasites on sheep properties are not ideal, and we have undertaken work to improve the assessment of these important disease organisms, in particular, assessing the amount of parasites from different species using a DNA-based method (qPCR). The tests can identify and quantify barber's Pole, small brown stomach and black scour worms in faeces samples. The tests have been rigorously evaluated and are ready for pilot usage by diagnostic laboratories and managers of sheep grazing properties.

Executive summary

The objective measurement of disease organisms in the environment and in animals will help livestock owners manage disease. The objective assessment of wool and meat quality has been important in livestock agriculture and has enabled increases in productivity. We believe that the management of animal health should also be objective and that this will lead to further increases in the efficiency of livestock production enterprises. The infectious organisms which cause most production loss in sheep production include the nematodes Haemonchus (barber's pole worm), Teladorsagia (small brown stomach worm) and Trichostrongylus (black scour worm) which all reside in the gastrointestinal tract but which result in different disease conditions and subsequent effects on production. Current methods for assessing gastrointestinal nematode parasites on sheep properties are not ideal, because the species cannot be separately quantified in egg counts (WEC), and the number of eggs present in faeces is not necessarily correlated with the number of larvae obtained from faecal culture (FCLD). We have undertaken work to improve the assessment of these important disease organisms, in particular, assessing the amount of parasites from different species using a DNA-based method (qPCR). The tests can identify and quantify barber's Pole, small brown stomach and black scour worms in faeces samples. The tests have been rigorously evaluated via the SCAHLS recommended process including comparing the results of qPCR with WEC and FCLD. These qPCR tests are now ready for pilot usage by diagnostic laboratories and managers of sheep grazing properties. A further increase in the number of tests conducted where comparison to WEC and FCLD is possible will assist in future quality assurance of the tests. In particular, further quality assurance work would be advisable for the small brown stomach worm qPCR test, which has undertaken less evaluation because of the smaller number of available samples containing this parasite. Furthermore, additional quality assurance using methods other than FCLD would be beneficial because of the poor relationship between FCLD and WEC.
Future research work should include a field trial to demonstrate the use of the qPCR tests in a commercial setting (including cost:benefit analysis), development of additional tests which can be undertaken using the same samples (liver fluke) or for different fractions from the same samples (host DNA – genotyping, parentage testing) and some scoping research to evaluate tests for important pathogens in cattle.

The SCAHLS evaluation work has also highlighted the need for a livestock parasite reference laboratory in Australia, the need for a larger collaborative network of livestock parasite diagnostic laboratories and more investment in parasite research and education. The existence of a reference laboratory at the centre of a diagnostic laboratory network would enhance our ability to develop diagnostics, evaluate new or re-emerging parasitic diseases, distinguish between endemic and exotic disease outbreaks, reverse the loss of skilled staff and support the training of parasitologists needed for laboratory and field-based extension and research work. The livestock parasitology research community has been undermined by sustained funding cut backs in multiple states of Australia and federally over the past two decades. This is a service which will be lost to the livestock sector unless larger investments in the area are made within the next two or three years.

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1 Background

In 2006, the cost of internal helminth parasites to the Australian sheep industry was estimated at AUS$369 million (Sackett and Holmes, 2006). Anthelmintics make up most of the ~AUS$50 million input cost component of the total cost and the sub-optimal use of anthelmintics results in a large proportion of the production loss which makes up the remainder. Current diagnostics for internal helminth parasites are imprecise and slow. Precise and timely diagnostics for these parasite diseases could provide valuable information for economically optimal decision making and use of anthelmintic drugs on farm (Gasser et al., 2008; Hunt, 2011; Hunt and Lello, 2012).

CSIRO and the University of Melbourne recently completed an MLA-funded project (Hunt and Gasser, 2009 - AHW.100) aimed at demonstrating the potential for DNA-based tests to replace traditional visual worm egg-count (WEC) and faecal larval differentiation (FCLD) methods used to diagnose intestinal helminth parasites in sheep. The project demonstrated that real time quantitative PCR (qPCR), a rapid and high-throughput DNA-based methodology, could be used to enumerate and speciate nematode eggs from faecal samples to a higher degree of precision and in a more timely manner than current methods (Bott et al., 2009). The methodologies developed did not increase the work-load or complexity of testing procedures for sheep producers because they utilised faecal sampling from animals in exactly the same way as in current farming practice. The quality of information returned to the producer however is enhanced by knowledge of species composition of infections assessed per sample at the same time as egg count. The tests can distinguish between species which cannot be easily separated by visual inspection of cultured larvae (for example benign Chabertia ovina vs highly pathogenic Oesophagostomum columbianum).

The tests developed allow a great deal of potential future enhancement, without altering current practice on farm. DNA extracted from faeces can be used to quantitate and/or identify a large range of pathogens in addition to intestinal nematodes. Additional organisms which could be identified in sheep and goats include lung worm larvae, liver fluke eggs, protozoan parasites, bacteria and viruses. In a separate project we have demonstrated the detection of liver fluke eggs in faeces using qPCR (McNally, 2013). The technology also can be used to analyse host DNA for quantity (may be associated with some pathologies) and composition (genotyping). Parasite and pathogen DNA could also be analysed in a compositional manner to detect for example drug resistance alleles and in-principle demonstration of this capability was undertaken within AHW.100. In addition, the technology is probably easily transferable to cattle, deer and other grazing animals. The tests in a modified form have also been used to detect parasitic larvae on pasture (Sweeny et al., 2012).

A key limitation to tests based on DNA extracted directly from faeces is the carry-over of contaminating substances into the DNA preparations which can inhibit PCR reactions. The project demonstrated two of three possible ways of avoiding problems associated with inhibitory contaminants. It was shown that eggs purified from faeces prior to DNA-extraction are relatively free from inhibitory contaminants, allowing speciation of eggs based on PCR to proceed. This method worked very well, but depended on visual egg counts for quantification and did not allow added tests to be carried out for organisms not co-purified with nematode eggs. The second method of avoiding inhibitory contaminants involved a number of steps to purify DNA prior to PCR. These methodologies worked to varying degrees and unfortunately some methods became unavailable during the course of the project. It was also discovered

that hydrolysis probe (Taqman) type qPCR reactions were less susceptible to inhibitory contaminants than SYBR green reactions. A third possibility for avoiding inhibitory contaminants would be to conduct non-enzymatic DNA quantification rather than qPCR or rolling circle amplification (another enzymatic method). Non-enzymatic methods were not explored in the project, though some of these are known to be less useful than qPCR for quantification (for example DNA-chip hybridisation).

The commercial application of tests developed as part of B.AHW.0100 required further work to streamline methodology, enhance DNA extraction methods, develop decision-making tools for evaluating results prior to communication with customers, evaluating methodology within a commercial diagnostics laboratory work flow and compare results to those obtained with existing methods. Development of key "add-in" features such as drug resistance testing, or testing for other non-nematode pathogens might also assist commercialisation by adding to the desirability of of the product to the end user (sheep producers). MLA has existing investments in developing DNA-based tests for some non-helminth pathogens of interest, and bringing these tests into a commercialisation strategy will be advantageous should they be available. The project described here concentrated on detection, speciation and quantification of Trichostrongylus, Haemonchus and Teladorsagia in suitably equiped diagnostic laboratories.

2 Project objectives

1) Enhance DNA extraction methods – New products and methodologies will be evaluated and assessed at the onset of the project.

2) Evaluate methodology within the SCAHLS framework – work, in collaboration with EMAI, to evaluate the multiplex nematode PCR tests for submission to the SCAHLS compendium of "tests in routine use"..

3 Methodology

Nematode eggs are found in the faeces so faecal samples gathered either from the ground or directly from the rectum are used. Samples are to be obtained fresh and preserved in ethanol, to a minimum of 50% by volume, within 3 hours of collection. 25 mL polystyrene collection vials are used for samples. Each vial should be filled to approximately half way with faeces irrespective of moisture content and ignoring air spaces between pellets. Ethanol is added after samples have been collected; at least 12.5mL ethanol is added to each vial.

Samples remaining in ethanol are stable for at least 6 months at room temperature. Samples can be transported by post, subject to flammable liquids regulations and can be sent at room temperature. No more than 500 mL of ethanol may be posted in each package. This constitutes 40 samples prepared as above.

Primer sets were designed to amplify multiple species of nematodes, in particular the Trichostrongylus primer which needed to amplify a number of closely related species, so contains four degenerate nucleotides. Probes were designed to match only one genus. Blastn analysis of the forward primers and probes was conducted to show their specificity (see SCAHLS document – MS2 report).

The nucleotide sequence of the primers and probes is:

Teladorsagia
Probe CCGTCGTAACGTTCCTGAATGAT
Forward primer GTTCAAGAATAACATATGCAAC

Haemonchus
Probe TGACATGTATGGCGACGATGTTC
Forward primer TCAAGAACATATACATGCAAC

Trichostrongylus
Probe CCTGTATGATGTGAACGTGTTGT
Forward primer BAGTTBAAGAAYAATAYATGCAAC

Universal reverse primer (NC2)
TTAGTTTCTTTTCCTCCGCT

A complete, detailed description of the DNA extraction and PCR protocol is attached (Appendix). Briefly the procedure is as follows:

On receipt in the laboratory, samples in ethanol are dried at room temperature in a fume hood (over night).

Dried samples are ground to achieve a consistency like coffee-grounds, this mixes the sample prior to sub-sampling, and the reduced particle size aids the addition of the subsample to tubes for the following steps.

A 0.5g sub sample is subject to bead beating (dry) with chrome steel balls (5 mm) and silicon carbide particles (1 mm) (2 minutes)

4.5mL of DNA extraction (=lysis) buffer is added and further bead beating is undertaken to ensure DNA is released into solution.

The sample is separated by centrifugation and 1 mL of supernatant (=lysate) is transferred to a 2 mL centrifuge tube with 0.1g PVPP and a further 1 mL of DNA extraction buffer.

Samples are incubated for 4 hours with shaking whilst impurities become adsorbed to the PVPP particles.

PVPP particles are separated by centrifugation and 600 µL of the supernatant is used to extract DNA using the Ambion magnetic-bead based DNA exrtaction kit using the Kingfisher automated DNA extraction equipment.

Extracted DNA is used for qPCR at 1/10 dilution.

qPCR set up as follows:

PCR setup:	x 1
template (1/20 diluted)	4
Hc forward primer (10µM)	0.5
Trich forward primer (10uM)	0.5
Telci forward primer (10µM)	0.5
NC2 reverse primer (10µM)	1.5
Hc probe (TET) (10µM)	0.2
Trich probe (TAMRA) (10µM)	0.2
Telci probe (FAM) (10µM)	0.2
2 x iQ™ Multiplex Powermix [a]	10
Milli Q water	2.4
Total	20µl

[a] Bio-Rad Laboratories iQ™ Multiplex Powermix. Catalogue number: 172-5849

1. PCR cycling conditions

Step	**Temperature (°C)**	**Time (minutes:seconds)**	**Number of cycles**
1	95	2:00	1
2	95	0:30	45
	56	1:00 (acquire fluorescence)	

Various experiments were conducted using this protocol to establish the diagnostic properties of the multiplex three-genus assay.

4 Results

Pilot experiments were conducted to optimise DNA extraction. The aim was to develop a procedure that could:

use whole faeces and avoid purification of eggs
extract DNA that was useful for qPCR
extract DNA using a quantitative procedure

Two routes of investigation were followed. First, data was generated to address the diagnostic properties of the test as required by SCAHLS. The acquisition of this data is described in full within the SCAHLS document (MS2 report). Secondly, some data analysis was undertaken of the correlation between microscopy-derived estimates of WEC and relative presence of the parasite genera and the qPCR results.

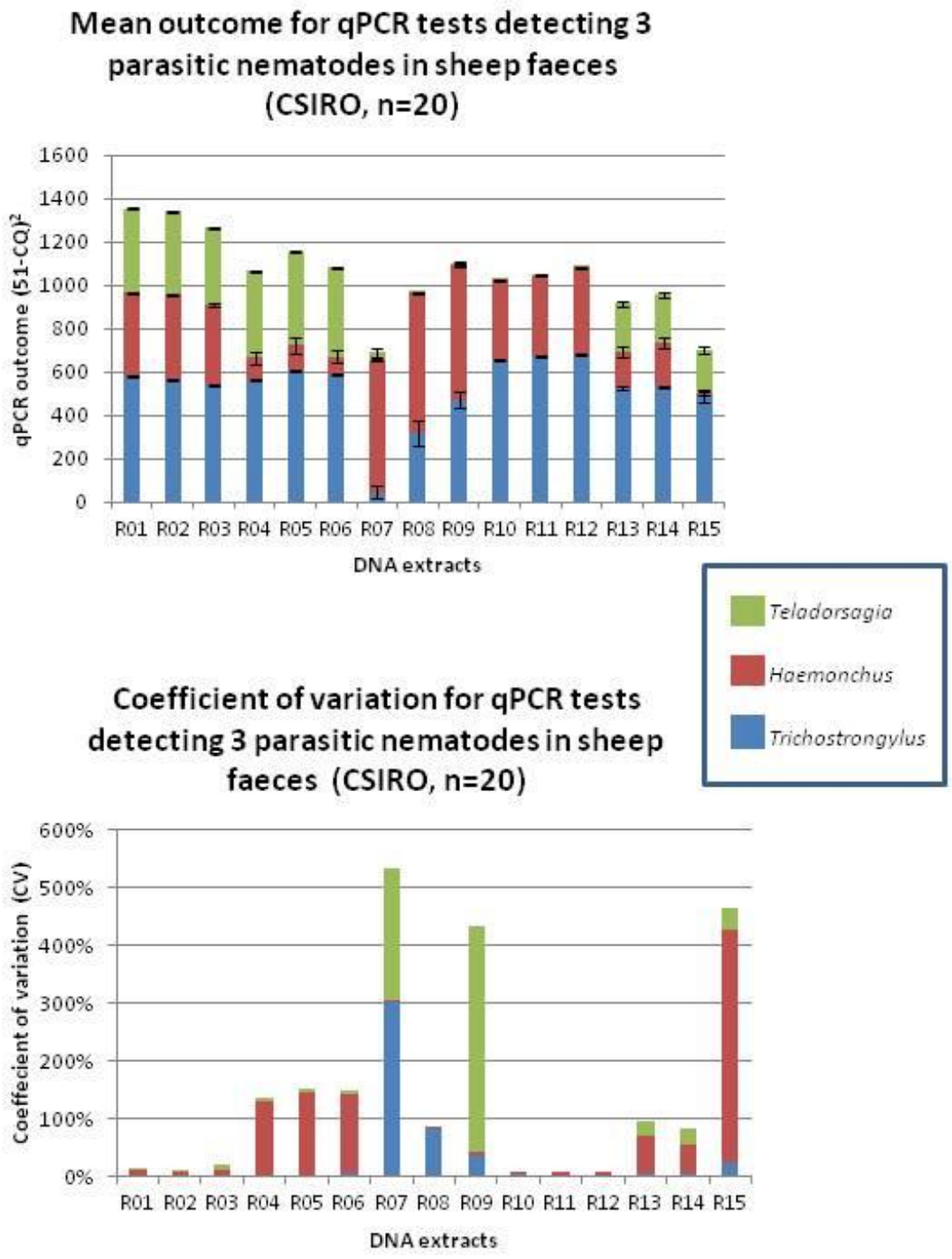
Section 1 – SCAHLS data

1.1 Repeatability

Five faecal samples were obtained from the rectum of five different sheep. A sub sample of the faeces from each sheep (approximately 4 g wet weight) was suspended in ethanol and the DNA extracted as described (Appendix).

Each DNA extract was used in 20 PCR amplifications using the multiplex qPCR test. Each amplification was undertaken in triplicate and the mean of the three replicates was used for further data analysis. Coefficients of variation between DNA extracts from the same faecal sample ranged from 0% to 350% (table below). The variation was far higher where the species was present at lower levels. This can be seen in the figure below the table. For example, compare results for sample three has very little Teladorsagia amplification, the over-all CV (across extracts) was 350%, but the genus was only detected in two of the three DNA extracts (R07, R09 but not R08), and the CV (within extracts) was high in both these.

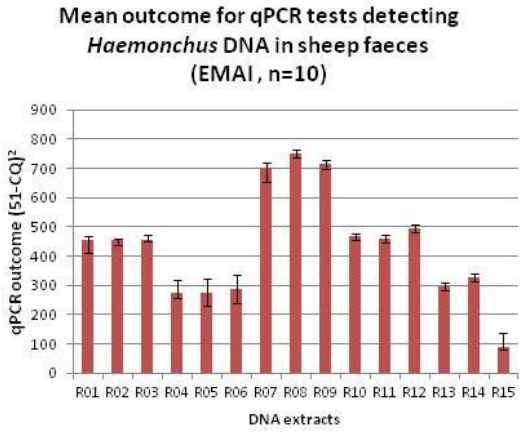
Faecal sample (DNA extract)	*Haemonchus*	*Trichostrongylus*	*Teladorsagia*	Total
Sample 1 (R01-R03)	7%	5%	7%	5%
Sample 2 (R04-R06)	136%	5%	6%	13%
Sample 3 (R07-R09)	5%	95%	350%	29%
Sample 4 (R10-R12)	6%	4%	0%	4%
Sample 5 (R13-R15)	98%	14%	29%	23%

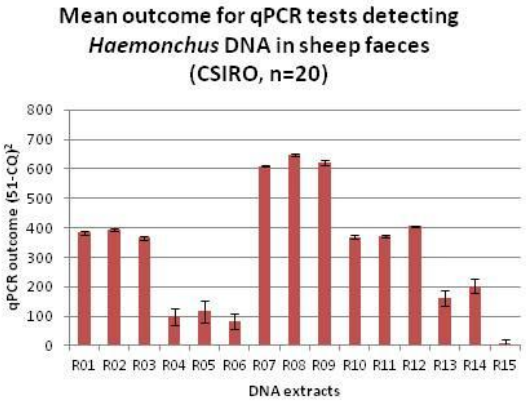


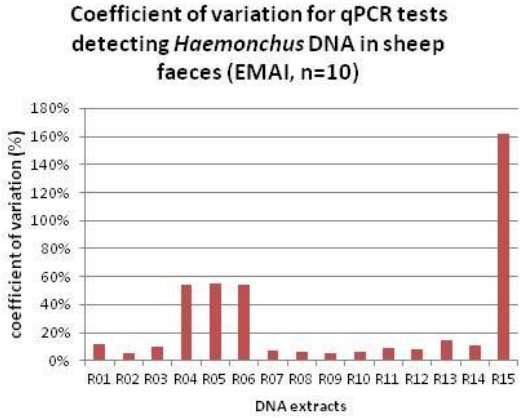
1.2 Reproducibility

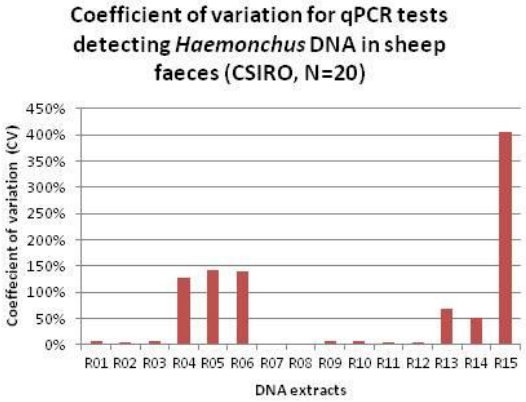
The fifteen DNA extracts were analysed by staff at the NSW DPI diagnostic laboratory at Menangle (the Elizabeth MacArthur Institute, EMAI). The qPCR instrument used was of a different make (ABI) to that used at CSIRO (BioRad), and the fluorophore for Haemonchus detection was changed to fit machine settings (CalGold rather than TET). qPCRs were repeated 10 times in triplicate. Means of the triplicate data were used to analyse repeatability of detection for the three genera of

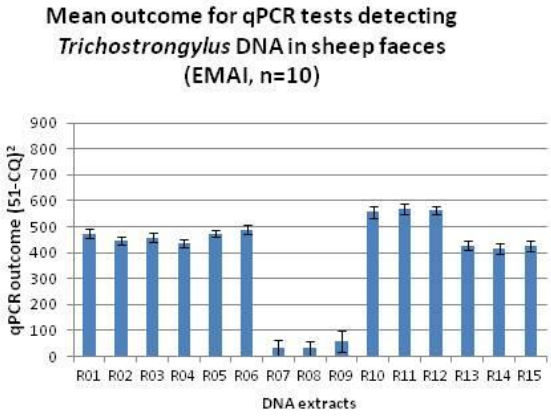
nematodes. The figures below show comparisons of the outcomes for the three genera when the qPCR was conducted at CSIRO or EMAI. The figures show the mean CQ (transformed by the formula (51-CQ)2)± SEM in the upper graphs and the corresponding coefficients of variation in the lower graphs. For Haemonchus and Trichostrongylus the level of detection and the amount of between and within extraction variation are similar between the laboratories. For Teladorsagia, the detection level at EMAI was higher, and this was especially evident with sample 3 (R07-R09). This may be because of an increased sensitivity at EMAI, though an alternative explanation of fluorophore overlap (between FAM and CalGold) has also been posed. At this time an explanation for the difference is not apparent.

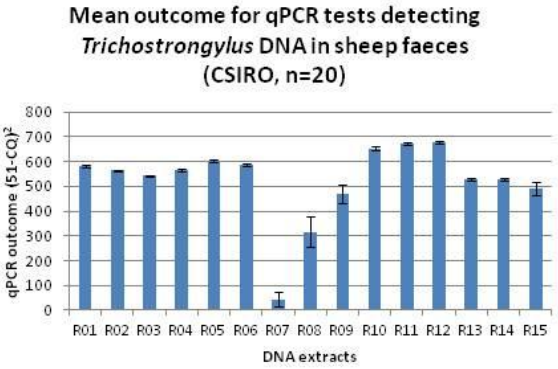


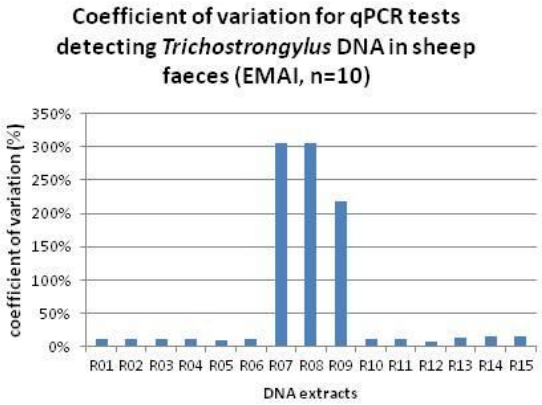


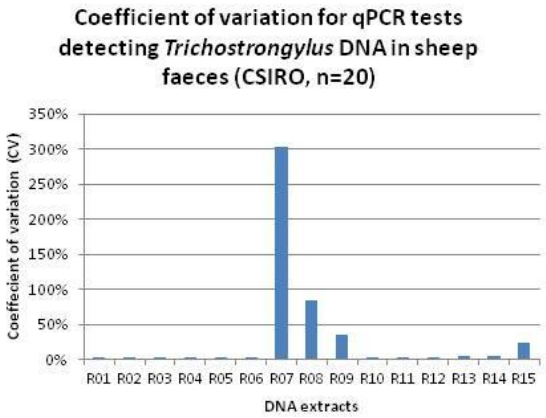


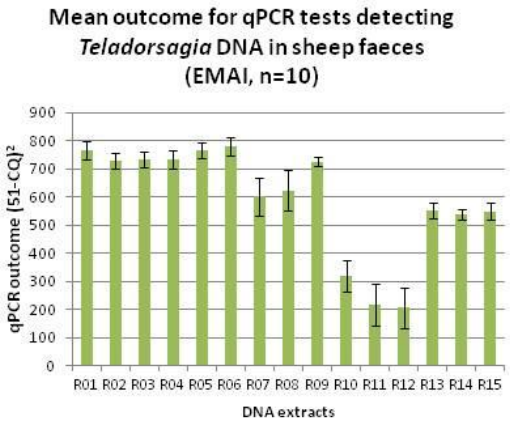


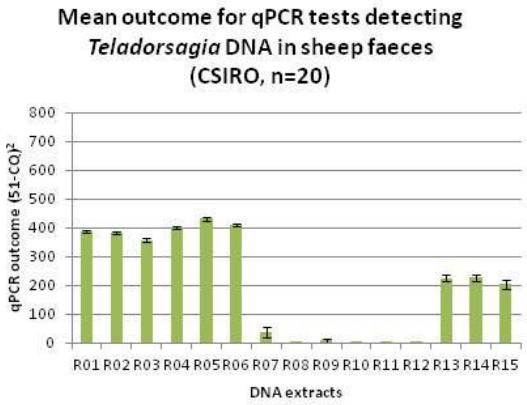

Mean outcome for qPCR tests detecting *Trichostrongylus* DNA in sheep faeces (EMAI, n=10)
qPCR outcome (51-CQ)²
900
800
700
600
500
400
300
200
100
0
R01 R02 R03 R04 R05 R06 R07 R08 R09 R10 R11 R12 R13 R14 R15
DNA extracts

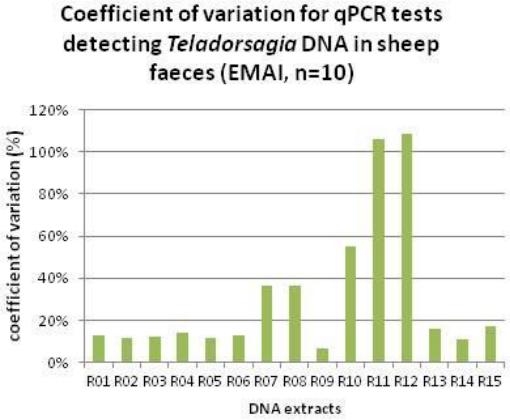

Mean outcome for qPCR tests detecting *Trichostrongylus* DNA in sheep faeces (CSIRO, n=20)
qPCR outcome (51-CQ)²
800
700
600
500
400
300
200
100
0
R01 R02 R03 R04 R05 R06 R07 R08 R09 R10 R11 R12 R13 R14 R15
DNA extracts

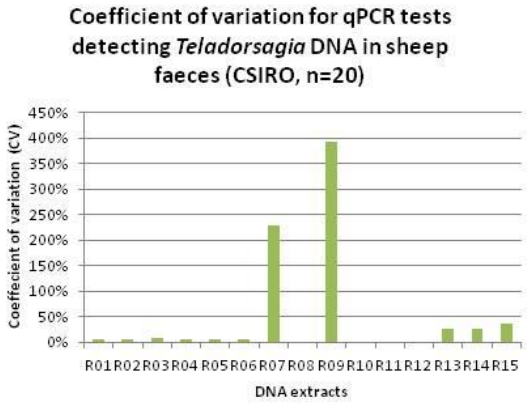

Coefficient of variation for qPCR tests detecting *Trichostrongylus* DNA in sheep faeces (EMAI, n=10)
coefficient of variation (%)
350%
300%
250%
200%
150%
100%
50%
0%
R01 R02 R03 R04 R05 R06 R07 R08 R09 R10 R11 R12 R13 R14 R15
DNA extracts


Coefficient of variation for qPCR tests detecting *Trichostrongylus* DNA in sheep faeces (CSIRO, n=20)
Coeffecient of variation (CV)
350%
300%
250%
200%
150%
100%
50%
0%
R01 R02 R03 R04 R05 R06 R07 R08 R09 R10 R11 R12 R13 R14 R15
DNA extracts









1.3 Range of detection

Field samples tested to determine sensitivity had levels of the defined five measures of infectious nematodes as follows:

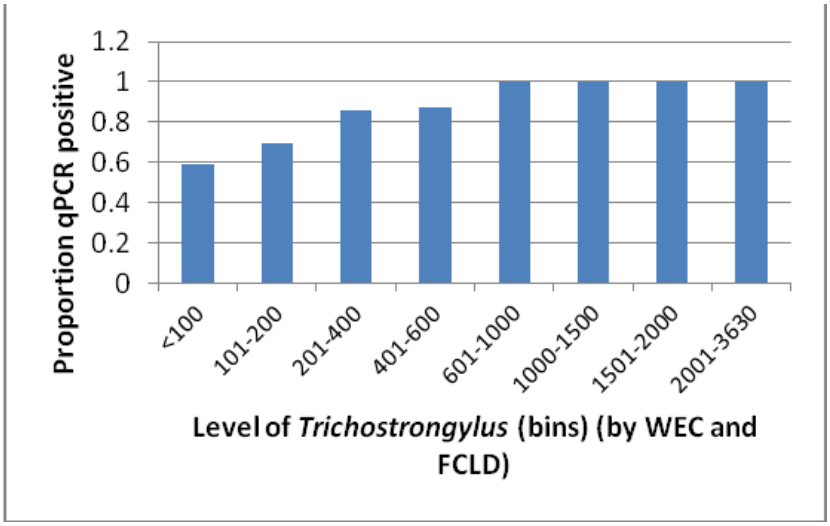
WEC	100 - 11,000 eggs/g
Hc WEC	64 - 8,159 eggs/g
TrTe WEC	1 - 3,960 eggs/g
Tr WEC	1 - 3,630 eggs/g
Te WEC	1 - 330 eggs/g

Upper limits of detection were not reached in the testing of field samples. The standards enable detection up to 10,000 eggs/g (*Teladorsagia*), and 50,000 eggs/g (*Trichostrongylus* and *Haemonchus*).

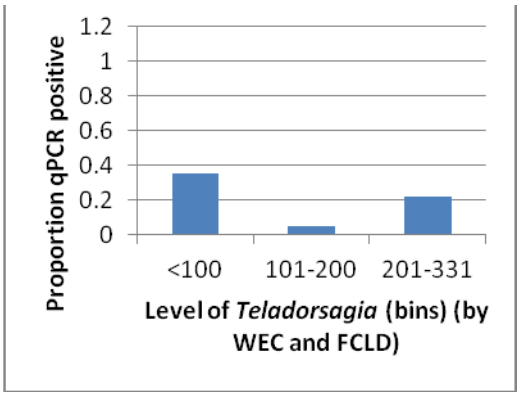
The lower limit detected for each of these measures was:

WEC WEC)	100 eggs/g (the limit of measurement employed for
Haemonchus WEC graph below	64 eggs/g (as calculated from FCLD and WEC) see
TrTe WEC	0.5 eggs/g (as calculated from FCLD and WEC)
Trichostrongylus WEC graph below	1 eggs/g (as calculated from FCLD and WEC) see
Teladorsagia WEC graph below	0.5 eggs/g (as calculated from FCLD and WEC) see

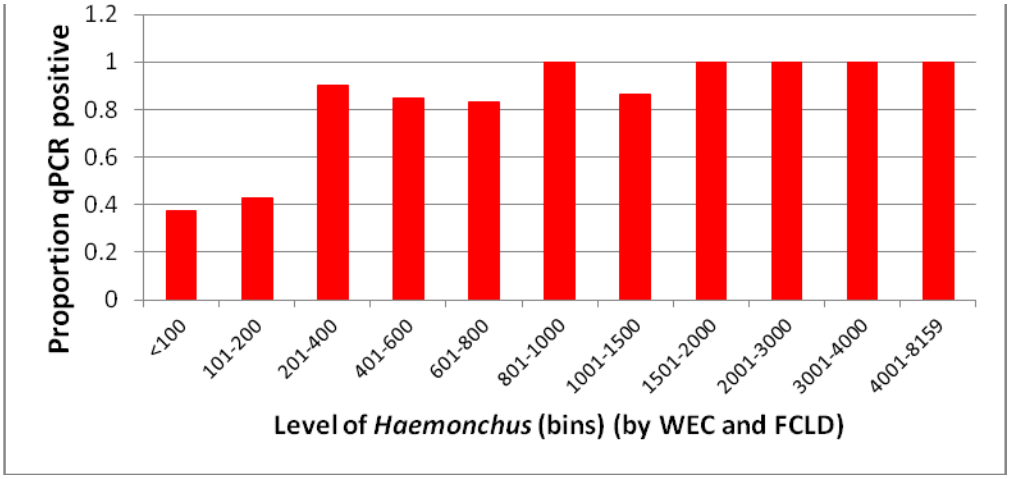
Reliability of detection of Trichostrongylus at differing levels of abundance (as measured by FWEC and FCLD)



Reliability of detection of Teladorsagia at differing levels of abundance (as measured by FWEC and FCLD)



Reliability of detection of Teladorsagia at differing levels of abundance (as measured by FWEC and FCLD)



1.4 Diagnostic sensitivity

Where the PCR result was positive, and presence of the nematode (or combination) was known within the sample as presented above, these values were defined as positive congruents. Where CQ_T = 1, and presence of the nematode (or combination) was known within the sample as presented above, these values were defined as false negatives. The ratio of positive congruents to the total number of positives is presented below as sensitivity.

	Trichostrongylus	***Haemonchus***	***Teladorsagia***	**WEC**	**Tr + Te**
Sensitivity	0.758	0.877	0.078	0.877	0.749
n	132	123	92	188	167

The sensitivity for *Teladorsagia* is not acceptable, so this assay as a stand alone test has not been used in further analyses. It is used as part of the WEC and TrTe calculations. The reasons for the low diagnostic sensitivity for *Teladorsagia* detection are not known, however the species parasitising sheep (*T. circumcincta*) has low prolificacy in combination with robust survival ability in the non-parasitic stages. It is possible that the level of eggs in the positive samples is much lower than would be expected given the results of FCLD. Further work is needed to establish the value of the *Teladorsagia* assay, a crucial requirement is field-derived samples containing much higher levels of *Teladorsagia* eggs, than were available from the three farms we accessed.

1.5 Diagnostic specificity

Low levels of parasitic nematode infection are common under Australian conditions for all ages of sheep. Though these infections pose no clinical risk, they make the use of field-grazed animals problematic for the investigation of diagnostic specificity.

We assembled a set of 25 animals, each derived from one of three treatments which aimed to produce truly uninfected animals.

The samples were from:

Helminth naive sheep: 7 sheep brought indoors within 24 hours of birth, before any oral experience of contaminated pasture could have occurred. The animals were then raised indoors on slatted floors which do not allow the accumulation of infected faeces, nor the development of eggs in the faeces into infective larvae. Faecal samples were obtained from the animals at 3-6 months.

Multi-drug treated sheep: 13 sheep brought indoors as described above and treated with multiple anthelmintics (Monepantel, albendazole, levamisole, praziquantel, oxfendazole, triclabendazole and abamectin). Animals were housed for two weeks prior to faecal collection.

Sheep 48 hours after drug treatment: 5 sheep were treated with Monepantel at the recommended dose. The animals were kept in outdoor yards with minimal exposure to contaminated forage for 48 hours before faecal samples were taken.

Where qPCR was negative, these values were defined as negative congruents. Where qPCR was positive these values were defined as false positives. The ratio of negative congruents to the total number of uninfected samples is presented below as specificity.

Specificity is high for all three assays and hence for the two calculated measures as well. These results provide evidence that there were no cross-reacting nucleic acids in the samples obtained from parasite free animals.

	CQ_{TTr}	CQ_{THc}	CQ_{TTe}	CQ_{TWEC}	CQ_{TTrTe}
Specificity	1.000	0.958	1.000	0.958	1.000
n	25	25	25	25	25

One sample gave a positive result for the *Haemonchus* assay, this sample was from one of the "*sheep 48 hours after drug treatment"*. It is likely this animal had not completely shed all the parasite material from its gastro-intestinal tract at the time of sampling. Experiments are underway to explore the time taken for qPCR-detectable nematode DNA to clear the GI tract after drug treatment.

Section 2 – Additional data

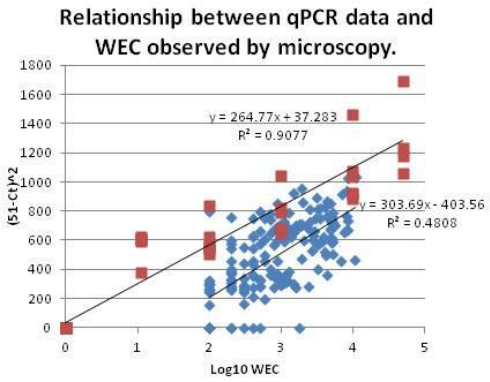
2.1 Quantitative relationships between qPCR and microscopy-based results

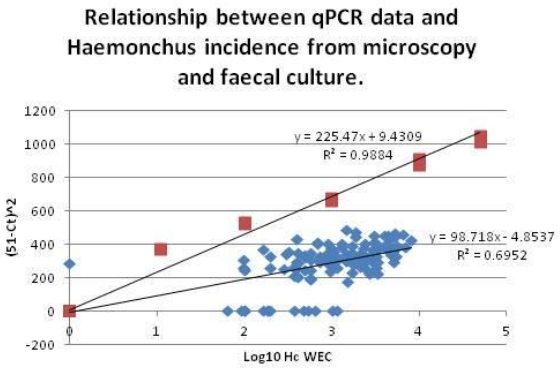
The table below shows R^2 values for the relationship between qPCR results and microscopy data. For WEC, this is the comparison between the sum of all three qPCR outcomes with WEC as determined by the standard McMaster salt flotation technique. For the individual genera, and for the combined *Trichostrongylus* and *Teladorsagia* data, the qPCR data are compared with WEC, multiplied by the proportion of nematodes from the genus amongst larvae observed after faecal culture.

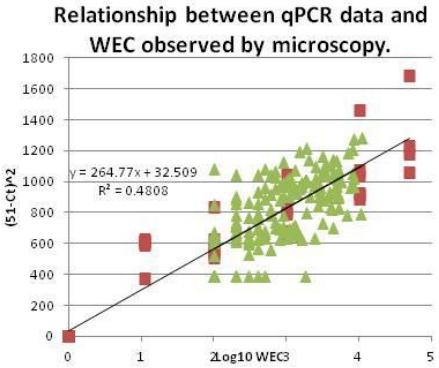
qPCR Test	R^2 (n for field samples)	R^2 for standards
Haemonchus	0.70 (133)	0.99
WEC	0.48 (161)	0.91
Trichostrongylus+*Teladorsagia*	0.10 (150)	0.95
Trichostrongylus	0.03 (144)	0.99
Teladorsagia	0.00 (36)	0.99

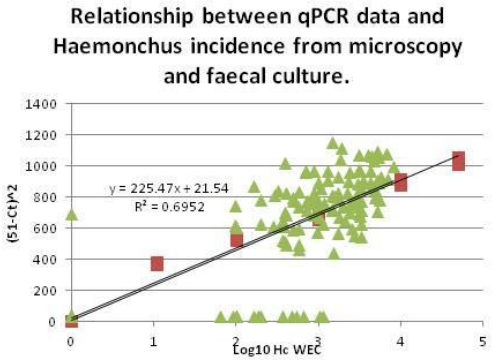
The table clearly shows that the relationship between *Haemonchus* larvae and *Haemonchus* qPCR is strong, and the relationship between total WEC and summated qPCR is also reasonable. The relationships for *Trichostrongylus* and *Teladorsagia* qPCR results compared to microscopy are low. Because the relationship between *Trichostrongylus*+*Teladorsagia* summed qPCR and the summation of the *Trichostrongylus* and *Teladorsagia* microscopy results is better than for either genus alone, it may be that there have been some errors in assigning the larvae from these genera based on morphology of third stage larvae. In addition, the relationship between summed qPCR results and WEC is reasonable, suggesting that the proportion of genera occurring as eggs in faeces differs from the proportions of the genera as estimated from larvae surviving laboratory culture. Both these possibilities suggest that further work more directly using eggs should be done to properly evaluate the *Trichostrongylus* and *Teladorsagia* qPCR tests. This might be achieved using the lectin binding assay developed by Dieter Palmer at DAFWA, though this can only distinguish *Haemonchus* from non-*Haemonchus* eggs. Other alternative against which the multiplex faecal qPCR tests might be evaluated would include qPCR using purified eggs (see AHW.100 reports), PCRs conducted on individual isolated eggs, or comparisons to total post-mortem worm counts. Though expensive, total post-mortem worm counts are the only parasite test regarded as a gold standard by other workers (e.g. the pharmaceutical industry).

Although the relationships between summed qPCR and *Haemonchus* qPCR with microscopy estimates are good, the data are not scaled the same as the standard curve. This is because of multiple factors, including differences in the way in which standards were prepared and field samples were processed, differences in moisture content between field samples and standards and qPCR efficiency differences between DNA samples derived from field-located or animal house sheep. The figure below shows qPCR data for standards (red squares) and field samples (blue diamonds), both compared to microscopy-based estimates. Using the regression solutions for the standards (above the upper linear regression line) and for the field data (beside the field sample regression line), data can be re-scaled (green triangles in lower graphs to allow read out of WEC data useful for field application.









Using WEC results based on the re-scaled data, we can re-estimate sensitivity and "specificity", based on various drench decision thresholds. This is a useful exercise to see how data could be used in farm management. The table below shows calculated values based on the data depicted above (for WEC).

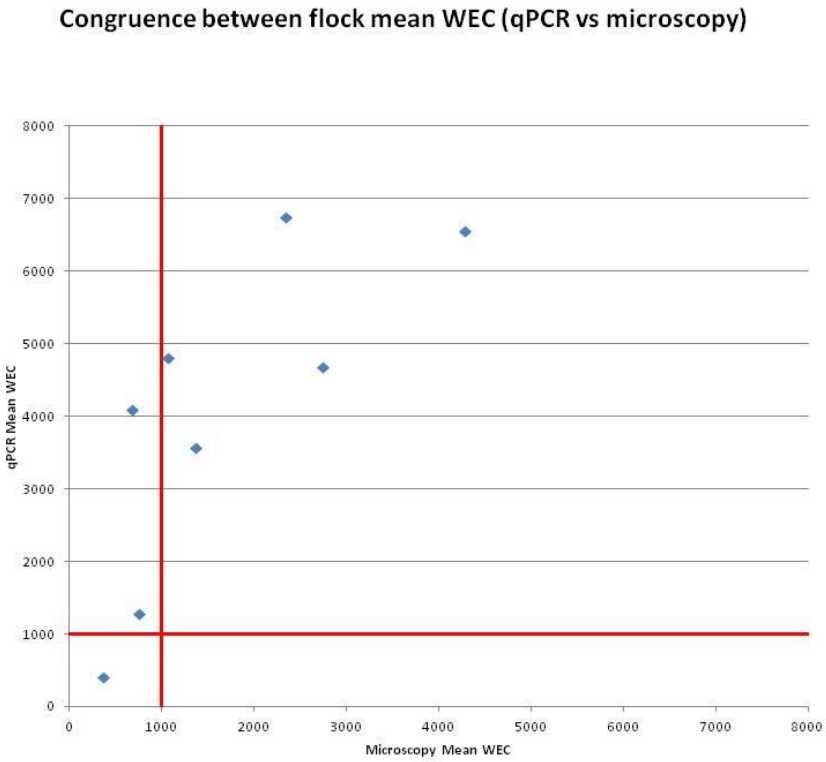
Drench threshold (eggs/g)	Sensitivity	Specificity
1,000	0.77	0.73
500	0.77	0.78
200	0.83	0.83

The data can also be depicted graphically. Drenching would be indicated for 22% of the samples by qPCR when it was not indicated by microscopy ("false positives) and for 23% of the samples drenching was indicated using microscopy results, but not when using qPCR results (false negatives).

For most on-farm drench decisions, the mean WEC between multiple samples obtained either from the rectum or the ground, is used. To investigate this type of data, the samples were grouped according to flock and date, and assigned to eight groups which had more than 5 sample data for both qPCR and microscopy. The table below shows the means for qPCR and microscopy, the number of samples compared (n) and the rank of samples using either technique. Given a 1000 eggs/g drenching threshold, the two samples were non congruent. Sample Ky2 has similar outcomes, but is close to the decision threshold for both methods. Sample Ky7 appears to have been either over-estimated using qPCR or under-estimated using microscopy. More work of this type will give further confidence for the interpretation of qPCR results. A useful experiment would be to run experiments where duplicate flocks were run and managed for parasites using microscopy and qPCR in parallel. Production and parasitological data comparisons would be made to investigate any

practical differences in the management of flocks. To make maximum use of the qPCR tests, prior knowledge of drench resistance (using FEC-RT), in particular the identification of species resistant to drug compounds would be sought. This would allow targeted drench choice for each drench decision, based on species presence. The value of obtaining this data from qPCR which is both a more direct and a faster method, could be evaluated in such a trial.

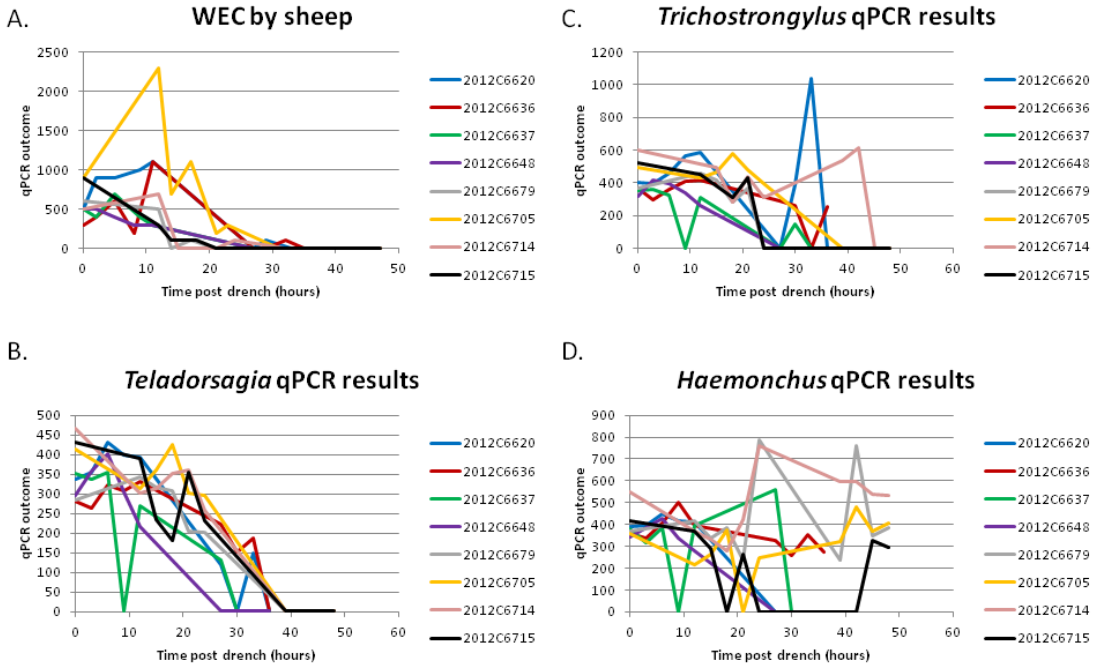
Group	Mean Calc FEC	Mean Actual FEC	n	Rank qPCR	Rank Microscopy
TSF1	6543	4286	37	2	1
Ky1	4672	2750	26	4	2
Ky6	6742	2350	6	1	3
Ky5	3563	1377	13	6	4
Ky4	4805	1076	17	3	5
Ky2	1268	763	8	7	6
Ky7	4093	689	9	5	7
TSF2	408	376	58	8	8



2.2 Detection of nematode DNA not derived from eggs

The detection of DNA derived from non-establishing infective larvae or from senescent adult parasites in faeces is a theoretical possibility. We conducted an experiment to investigate these possibilities. We took eight hogget wethers, infected at pasture, and introduced them to our animal house. The sheep were fitted with

faecal collection harnesses so that faeces could be collected for time intervals and processed for WEC and qPCR. The sheep were drenched, and faecal material collected at the time of drenching (time 0) and at each of twelve timepoints after the sheep were drenched. The figure below shows the timecourse of dissapearance of nematode eggs in faeces for each sheep (A. assessed by microscopy), and changes over time of qPCR detected parasites (B-D, arbitrary units) for each sheep. Although the disapearance of Teladorsagia DNA parallels the disappearance of observable eggs, the level of infection was likely low, these parasites are small, and the chance of dead worms remaining intect from the abomasum through to the rectum seems less likely than for worms residing more posterior in the gastrointestinal tract. In contrast, for both Haemonchus and Trichostrongylus the qPCR signal rises again to a peak between 24 and 48 hours after drenching. The simplest interpretation of these results is that qPCR has detected senescent adult parasites as they are excreted fro the gastrointestinal tract post drenching. In practical terms, it would be unwise to use these qPCR tests to infer WEC within days of drenching. Although it seems unlikely that a producer would attempt to do this, it is worth stating the limitations of the technique.



5 Discussion/conclusion

Major Findings

1) qPCR tests for Haemonchus and Trichostrongylus are validated and ready for use by diagnostic laboratories and their customers. The SCAHLS process may require additional laboratories to become involved, but the diagnostic properties of the tests have been proven sufficient for this next step.
2) qPCR test for Teladorsagia is ready for use but requires more validation to generate sufficient data. The SCAHLS process will require additional laboratories to become involved, to ensure the diagnostic properties of the tests can be proven sufficiently.
3) Alternative validation against other tests would be worthwhile because the technical variations which effect the relationship between WEC and FCLD are significant. The lectin binding assay, comparison to worm count data and

comparison to the faecal occult blood assay are other tests against which the qPCR test should be compared.

Further actions recommended

1) Continued support for the process of establishing the parasite detection faecal qPCR tests in state laboratories. This would include funds for sample sharing and collection, trials with multiple makes of qPCR machines and combinations of fluors. (EMAI, DAFWA and CSU are amongst the potential collaborators).
2) One or more field trials to establish the value of using the more direct and faster qPCR tests over the microscopy equivalents. Ideally both for faecal egg count reduction tests of drench resistance and for subsequent flock management. It would be appropriate to conduct these on both research properties and commercial grazing properties to obtain a balance between commercial setting and comprehensive assessment.
3) The presence of additional metazoan parasites of importance in the Australian sheep industry is known. Additional tests are required to complement the qPCR tests developed in this project. A project to develop these would be a sensible next step. Pilot work in our lab on *Fasciola* detection in sheep has been very encouraging, and would necessarily become part of continued test development work. Other priorities might be:
    a. Cooperia, Oesophagostomum and Chabertia for sheep
    b. Fasciola, Cooperia, Ostertagia, Haemonchus and Oesophagostomum for cattle
    c. Validation of sheep tests for use by goat producers
    d. Use of protozoan tests using the DNA extraction procedures we have developed (collaboration with Murdoch university)

4) Additional evaluation of tests, especially the *Teladorsagia* test is needed. The use of additional non-qPCR tests for comparison is required. Lectin binding assays, standard PCR with individual eggs or larvae and post mortem worm counts are possibilities. (DAFWA is ideally placed to collaborate with us toward this work).
5) Australia needs a national reference laboratory for livestock helminth parasites. A review and an action plan is needed to establish such a laboratory, define a support network for parasite diagnostic laboratories more generally and also to ensure support for training and education in livestock parasitology. We are poised to lose much traditional expertise in this area nationally and this loss will make the incorporation of modern techniques in animal disease management (e.g. new diagnostic technologies including qPCR) difficult or impossible.

Appendix

Test Method Protocol for Multiplex Nematode PCR for Inclusion in the ANZSDP for Sheep Nematode Disease

1. Reagents:

Sample Collection:
25ml polystyrene collection vials (eg Sarstedt – Cat No. 60.9922.113 PS)
Ethanol absolute

Sample Preparation
Filter papers Grade 42 diameter 90mm (ashless, 2.5uM pore size)
7ml screw cap polypropylene vials
1.0mm silicon carbide sharp particles (Daintree Scientific cat no. 11079110sc)
3.2mm Chrome-steel balls (Daintree Scientific, cat no. 11079132c)
2ml microfuge tubes (good quality eg Eppendorf safe-lock tubes)
MagMAX™ lysis/binding concentrate (Ambion Cat No. AM8500)
Poly(vinylpolypyrrolidone) ~110µM particle size (CAS No. 9003-39-8)

DNA extraction and storage
MagMAX™-96 Viral RNA Isolation Kit (Ambion Cat No. AM1836)
MagMAX™ Wash Buffer 1 (Ambion Cat No. AM8504)
MagMAX™ Wash Buffer 2 (Ambion Cat No. AM8640)
KingFisher deep well plates (ThermoScientific Cat No. KNG95040450)
King Fisher tip comb deep well (ThermoScientific Cat No. KNG97002534)
Kingfisher plate 200µL (ThermoScientific Cat No. KNG97002540)
8-tube strips, 0.2ml
96 well plates

qPCR assay
reduced-EDTA TE buffer (10mM Tris-Cl, 0.1mM EDTA, pH 8.0)
DNA LoBind safe-lock tubes 1.5ml (Eppendorf)
96 well plates (compatible with qPCR cycler)
iQ™ Multiplex Powermix (BioRad)
TaqMan dual coloured probes
PCR grade DNA oligonucleotides

2. Equipment/platforms:

50ml tube racks
Small plastic funnels (approx 7cm diameter)
Fume cupboard
Small grinder
Metal spatulas
BioSpec Mini-beadbeater-96 (Daintree Scientific – cat no 1001EUR)
7ml tube rack with microplate footprint (Daintree Scientific – cat no 504VH12)
Mini-Centrifuge
Horizontal shaker for microfuge tubes
Centrifuge – microfuge
Kingfisher-96 FLEX (Thermo Fisher Scientific) including BindIt software
Real time PCR machine (we used: BioRad iCycler 5)

3. Safety/bio-safety precautions/special laboratory requirements

The laboratory needs to meet minimal laboratory standards under the AS/NZS standards for design. To minimise contamination separate areas are needed for sample drying and grinding to the other steps of processing. The DNA and PCR should be handled and setup in a clean area of the laboratory. General laboratory procedures to minimise contamination should be undertaken such as wearing gloves and lab gowns as well as keeping the area clean and free of faecal materials. Regular decontamination of the work areas is necessary with bleach and/or ethanol including cleaning the grinder between samples with ethanol.

4. Test procedures: DNA Extraction from Sheep faeces.

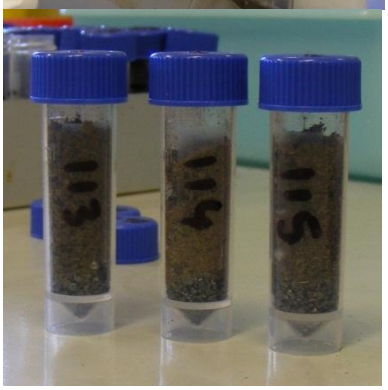
Ethanol preservation & sample preparation.

1. A fresh faecal sample is preserved in ethanol (denatured) in collection container, cover sample with ethanol. Sample usually left in ethanol at least overnight before drying.
2. Setup a funnel (diameter of approx 7cm) with filter paper (#42, 9cm circle) for each sample into a 50ml tube rack (or the like) inside a plastic container to collect any flow through. Ensure funnel tips are not touching the bottom of the rack to allow good air flow. Pour sample through filter carefully, not letting sample to over flow filter. Leave to air dry O/N in the fume cupboard. Remaining sample (if any) can have ethanol added to preserve it in case it is required.

Sample homogenisation – grinding and bead beating.

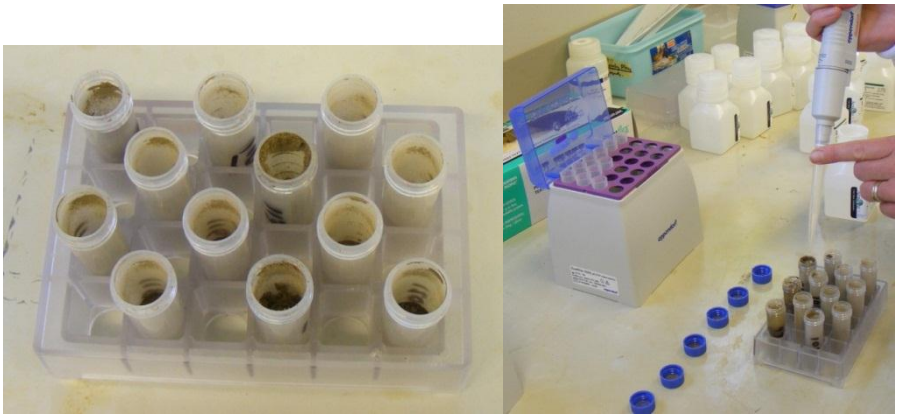
1. Pre-prepare a 7ml bead beating tube for each sample with the following, numbering both the tube and lid with relevant sample numbers:
    a. 2g silicon carbide sharp particles (1mm diameter)
    b. 2 x chrome steel balls
2. Grind each sample by adding it to a small grinder (eg. electric Breville grinder) and grinding for approx 5-10 seconds until sample is finely ground. Tare the balance with the corresponding tube on the pan. Weigh out 0.5g of ground sample into the pre-prepared bead beating tube.





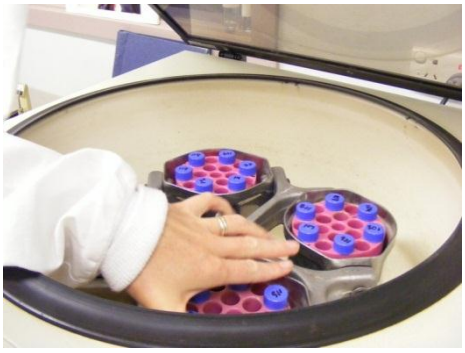
3. Wash the grinder by spraying with ethanol. Rinse the grinder with ethanol and drain out, repeat if necessary. Then spray the grinder and lid with ethanol and wipe out to dry. This is to prevent cross contamination. Ensure ethanol has evaporated from grinder before adding next sample for grinding.
4. 'Dry' bead beat the samples for 2 minutes in a BioSpec Mini-bead beater-96. Remove rack from bead beater and bang the rack on the bench to encourage samples to fall to the bottom of the tubes.


5. If a sample is at the top of the tube when you open its lid use a sterile wooden toothpick to poke it down, careful not to allow sample to spill.
6. Keeping the tubes in the rack, promptly remove each lid and add 4.5ml lysis/binding concentrate (Ambion). (another rack of samples could be run in the bead beater during this time, however do not leave dry bead beaten samples too long before adding lysis buffer to preserve DNA integrity)



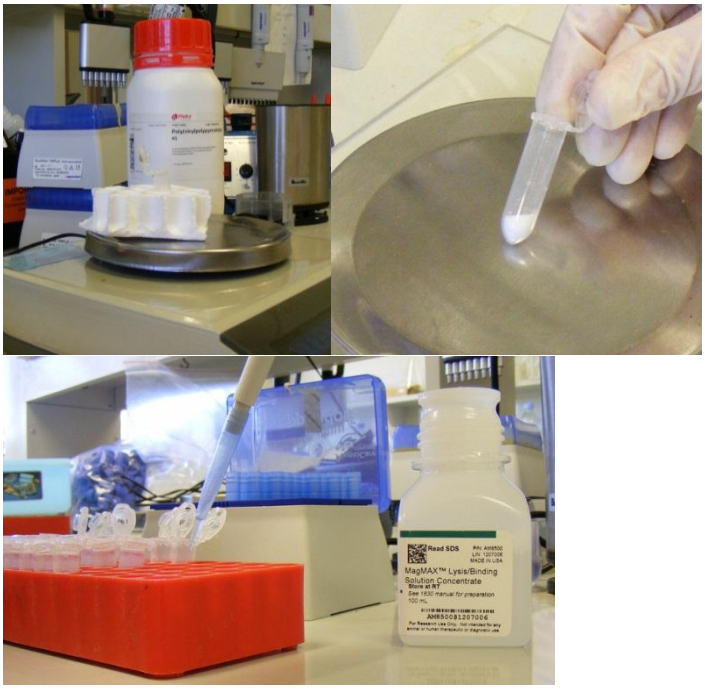
7. Tubes containing lysis/binding buffer are then ‘wet’ bead beat in the Mini-bead beater-96 for 2 minutes.

8. Remove tubes from the rack and centrifuge to separate beads and debris from the supernatant, 3000rpm 15 mins.

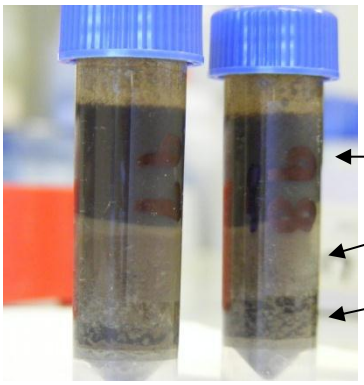


PVPP treatment

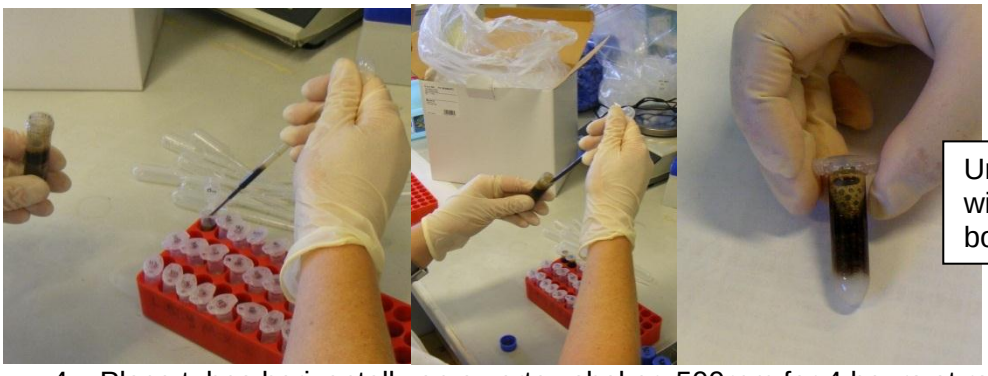
1. Label and prepare a 2ml microcentrifuge tube for each sample with 0.1g PVPP and 1ml lysis/binding buffer. Give the tubes a quick spin on a mini-centrifuge to ensure no air gaps are present.



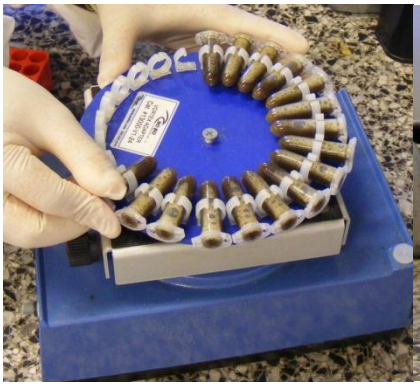
2. Remove tubes from the centrifuge. See picture below for layers visible.



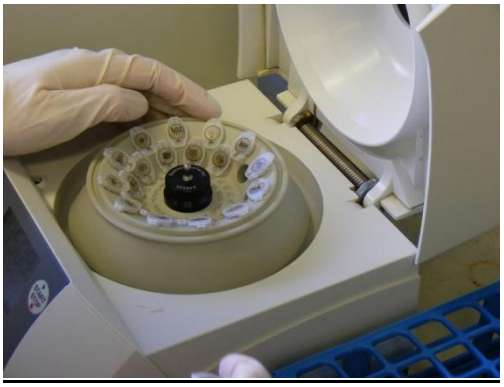
3. Remove 1ml of lysate from each centrifuged sample, add to the corresponding PVPP tube, invert a few times to mix PVPP through.



4. Place tubes horizontally on a vortex shaker, 500rpm for 4 hours at room temp.

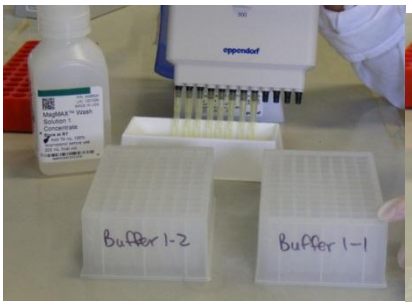



5. Spin PVPP out of the sample in bench top centrifuge at 10 000g 10mins.

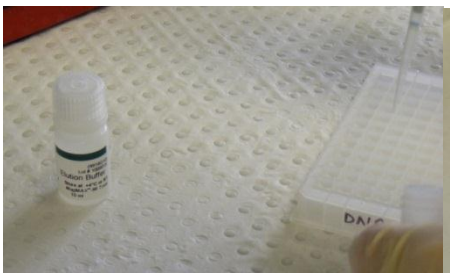


DNA extraction – Ambion MagMAX kit

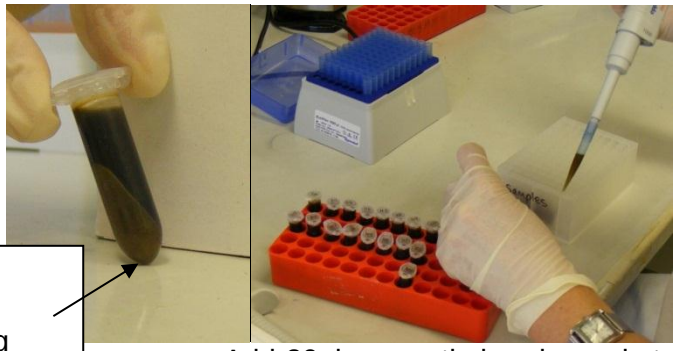
1. Prepare deep well plates for Kingfisher:
    a. Sample plate : 360µl isopropanol
    b. Wash 1-1 : 400µl wash buffer 1
    c. Wash buffer 1-2 : 400µl wash buffer 1
    d. Wash buffer 2-1 : 450µl wash buffer 2
    e. Wash buffer 2-2 : 450µl wash buffer 2



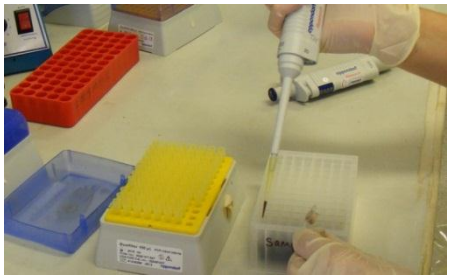
2. Prepare 96 well shallow plates for Kingfisher:
    a. DNA elution plate : 50µl elution buffer
    b. Tip comb plate



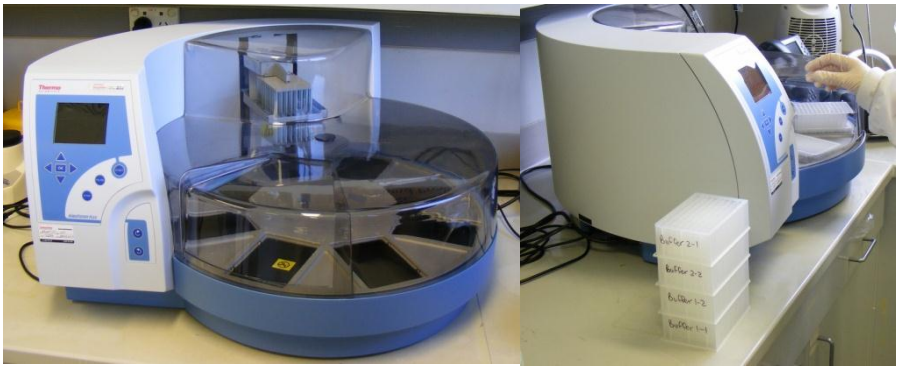

3. Prepare a master mix tube of magnetic beads – each sample needs 10µl beads + 10µl binding enhancer, allow 10% extra volume when making up master mix. (eg. For 24 samples add to master mix tube 264µl beads + 264µl binding enhancer) Beads must be vortexed and mixed before dispensing. Keep bead premix on ice until needed.
4. Add 600µl of sample supernatant (from centrifuged PVPP step above) to the sample plate. Ensure the location of the sample in the plate is tracked.

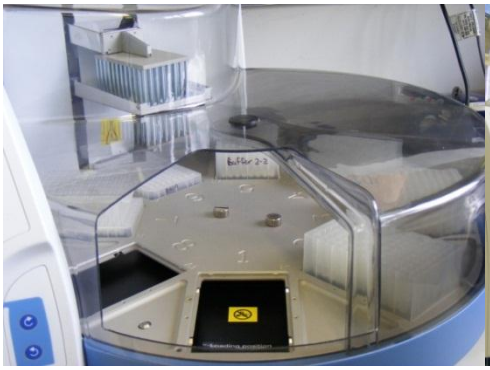


5. Add 20µl magnetic bead premix to each sample. Mix the premix before dispensing and by pipetting up and down during aliquoting to ensure that the beads are mixed evenly.

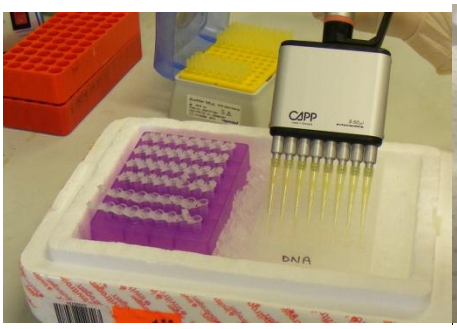


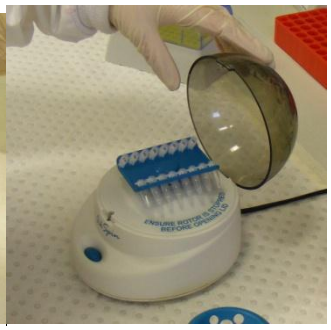
6. Open 'Faecalextract' program in Kingfisher FLEX (information about uploading program in Appendix below) Load Kingfisher with plates in the order it requests.






7. Remove eluted DNA solution from the Kingfisher promptly after it finishes and place the samples on ice.
8. Remove the DNA into individual 1.5ml tubes, strips of 8 tubes or a 96 well plate (whichever is more convenient for workflow). DNA samples need to be kept cold at all times and minimise freezing and thawing. Give samples a quick spin to ensure liquid is at bottom of tube/well before proceeding.





9. Working dilutions are made using reduced-EDTA TE buffer (10mM Tris-Cl, 0.1mM EDTA, pH 8.0).

<u>Preparation of primers and probes.</u>
Primer and Probe sequences are below. The reverse primer, NC2, is common to all the assays.

Species		**Oligonucleotide sequence**	**Amplicon Size (bp)**	**5' probe label**	**3' probe label**
Haemonchus	Forward	TCAAGAACATATACATGCAA	223		
(Hc)	Reverse	NC2 -			
	Probe	TGACATGTATGGCGACGAT		TET	BHQ1
Telidorsagia	Forward	GTTCAAGAATAACATATGCA	233		
(Teli)	Reverse	NC2			
	Probe	CCGTCGTAACGTTCCTGAAT		6-FAM	BHQ1
Trichostrongylus	Forward	BAGTTBAAGAAYAATAYATG	229-230		
(Trich)	Reverse	NC2			
	Probe	CCTGTATGATGTGAACGTGT		TAMRA	BHQ2

1. All stocks of primers and probes are stored in small aliquots to reduce freeze/thawing and reduce chances of contamination.

2. All primers and probes, working and stocks, are made up with reduced-EDTA TE buffer.
3. All TaqMan probes are kept away from light and kept on ice.
4. Stock solutions are 100µM and working solutions are 10µM.

PCR setup

1. DNA samples are diluted to 1/20 with reduced EDTA TE buffer for the working template (eg 3µl DNA plus 57µl reduced EDTA TE buffer).
2. Prepare a master mix comprising all components except the template (keep on ice). To calculate the quantities required of each component multiply the x1 amount below by for the number of reactions required plus approximately 5% to ensure there is enough mix for all wells.
3. Mix the master mix well then aliquot 16µl into each well required.
4. Add 4 µl of template to appropriate wells.
5. Controls included on each plate are: positive control for each of the three genera; a negative faeces control and a no template control (NTC).

PCR setup:	x 1
template (1/20 diluted)	4
Hc forward primer (10µM)	0.5
Trich forward primer (10uM)	0.5
Telci forward primer (10µM)	0.5
NC2 reverse primer (10µM)	1.5
Hc probe (TET) (10µM)	0.2
Trich probe (TAMRA) (10µM)	0.2
Telci probe (FAM) (10µM)	0.2
2 x iQ™ Multiplex Powermix [a]	10
Milli Q water	2.4
Total	20µl

[a] Bio-Rad Laboratories iQ™ Multiplex Powermix. Catalogue number: 172-5849

PCR profile:

Step	**Temperature (°C)**	**Time (minutes:seconds)**	**Number of cycles**
1	95	2:00	1
2	95	0:30	45
	56	1:00 (acquire fluorescence)	

Uploading Kingfisher method file

1. Connect a computer with BindIt software to the Thermo Fisher Scientific 'Kingfisher FLEX' using the USB data cable.
2. Save the faecalextract_KF.msz file provided onto the computer.
3. Open BindIt software
4. Turn on Kingfisher FLEX
5. Choose 'connect' in software and select active Kingfisher machine.

6. Choose ‘Import’. Locate the msz file using the browse option and import it to BindIt.
7. Choose ‘transfer’ and click on the ‘Backups’ tab. The faecalextract protocol should appear on the left hand box.
8. Select the protocol and then use the arrows pointing to the right to move it to a folder of choice on the right hand side.

5. Calibration required

Calibrate the qPCR machine as required by the manufacturer using the fluorescence spectra covered by the TaqMan probes used.

6. Specimen Collection and handling and treatment

Nematode eggs are found in the faeces so faecal samples gathered either from the ground or directly from the rectum are used. Samples are to be obtained fresh and preserved in ethanol, to a minimum of 50% by volume, within 3 hours of collection. 25 mL polystyrene collection vials are used for samples. Each vial should be filled to approximately half way with faeces irrespective of moisture content and ignoring air spaces between pellets. Ethanol is added after samples have been collected; at least 12.5mL ethanol is added to each vial.

Samples remaining in ethanol are stable for at least 6 months at room temperature. Samples can be transported by post, subject to flammable liquids regulations and can be sent at room temperature. No more than 500 mL of ethanol may be posted in each package. This constitutes 40 samples prepared as above.

7. Test acceptance criteria

Negative control - NTC - 2 µL PCR grade water added to qPCR mix in place of DNA template.

Negative control – Nematode free faeces - 2 µL template DNA (1/20 dilution) prepared using faecal sample from an uninfected sheep

Positive control – SPUD – From Nolan *et al* (2006) – 125pg SPUD amplicon DNA

Positive control – PPC-Ha - *Haemonchus* ITS2 plasmid – 240 fg plasmid DNA in 2 µL added in place of template

Positive control – PPC-Tr - *Trichostrongylus* ITS2 plasmid – 240 fg plasmid DNA in 2 µL added in place of template

Positive control – PPC-Te - *Teladorsagia* ITS2 plasmid – 240 fg plasmid DNA in 2 µL added in place of template

A negative result from any of the three plasmid positive controls or the SPUD control is an indication of assay failure. Where the SPUD positive control assay yields the expected result and any plasmid control does not, this is an indication of an issue with probes or primers in the MASP assay. Where the SPUD assay fails, there is likely to be a problem with the master mix.

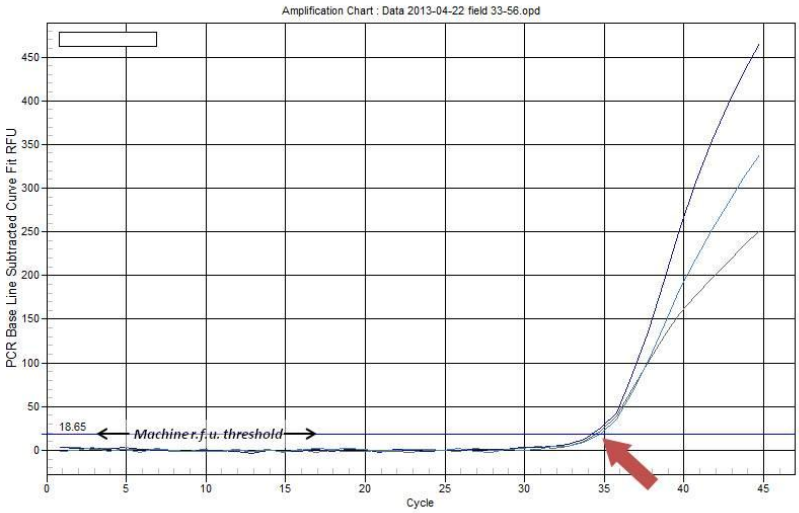
A positive result in the NTC and/or the NFF is also an indication of assay failure. It is most likely that contamination of PCR reagents with template has occurred. A positive result in the NFF assay where the NTC is not positive may also be indicative of contaminated DNA extraction apparatus.

Thresholds are used to evaluate very low readings in test samples. The examination of a regression analysis of standards and knowledge of the repeatability of qPCR assays was used to set a false positive threshold.

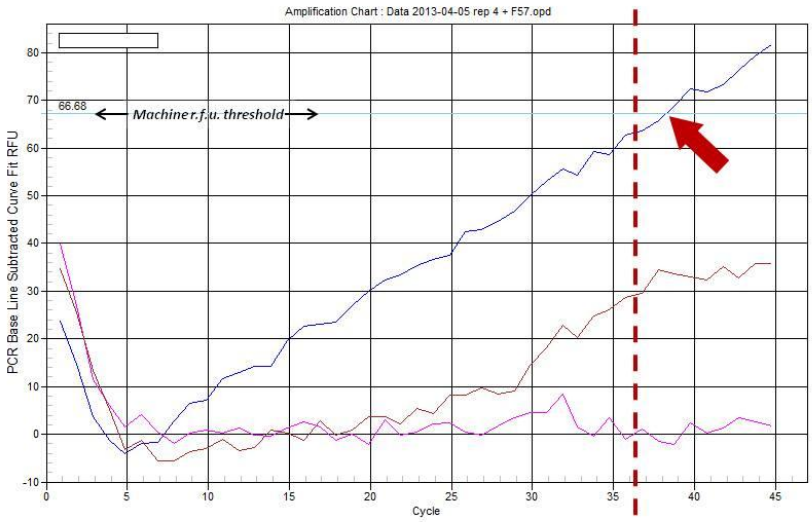
	Trichostrongylus	*Haemonchus*	*Teladorsagia*
Standard curve intercept	36.30	35.29	36.94
Mean Standard deviation of repeat data	0.66	1.00	1.01
Maximum CQ False positive threshold	36.96	36.29	37.95
Minimum CQ False positive threshold	15.77	18.40	20.51

Secondly, background fluorescence can sometimes rise gradually throughout the qPCR procedure. These curves, see figures below, are not typical amplification curves, and are easily distinguished from true qPCR detections by implementing an endpoint relative fluorescence (ERF) false positive threshold in addition to the Maximum CQ false positive threshold. In the data described herein, the ERF false positive threshold was set at 70 relative fluorescence units (r.f.u.).

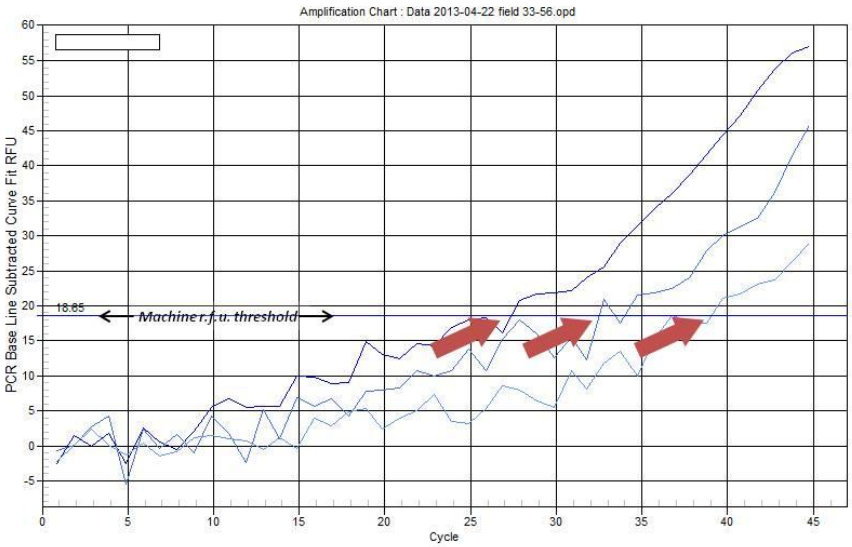
Fluorescence reaches threshold under the Maximum CQ threshold.
Typical curve shape. 3/3 technical replicates positive
(TAMRA probe – *Trichostrongylus* reaction)



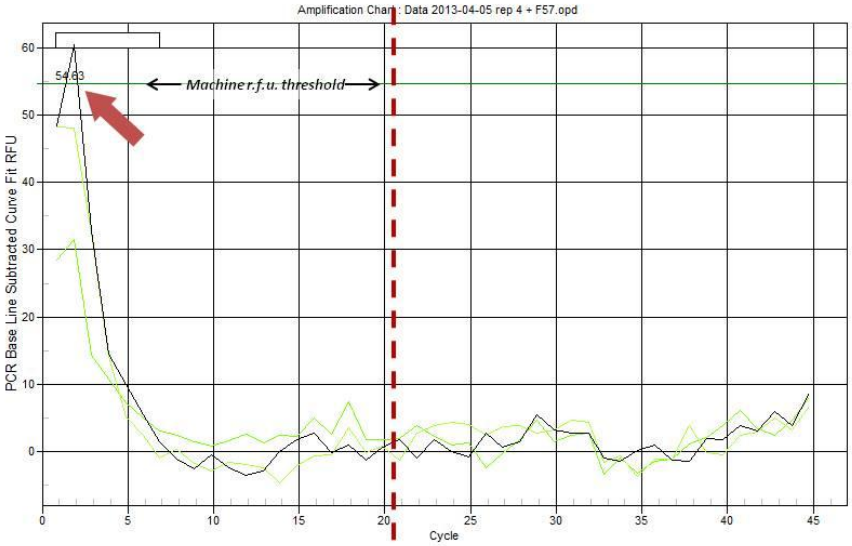
Fluorescence reaches threshold late in reaction (red arrow) and non-typical curve shape.
Maximum CQ threshold of 36.29 prevents false positive reading (Red dashed line).
(TET probe – *Haemonchus* reaction)



Non-typical curve shape. EPF threshold of 70 r.f.u. prevents false positive reading (TAMRA probe – *Trichostrongylus* reaction)



Fluorescence reaches threshold very early in reaction (red arrow) and non-typical curve shape. Minimum CQ threshold of 20.51 prevents false positive reading (red dashed line). (FAM probe – *Teladorsagia* reaction)



Thirdly, some reactions have an early peak of fluorescence which exceeds the machine's fluorescence threshold very early. These reactions are also false positives. To prevent these false positive data, a minimum CQ threshold was also enforced. The threshold was set at the minimum CQ reading obtained from the examination of standards less 0.1 (50,000 eggs/g for *Trichostrongylus* and *Haemonchus*, 10,000 eggs/g for *Teladorsagia*).

8. Result Interpretation

The qPCR test provides three replicate assessments of the cycle number at which PCR reaction fluorescence exceeds a threshold above background (C_Q). The threshold is usually assigned by the manufacturer's software, but can be adjusted manually. In accordance with guidelines suggested by the Australian measurement laboratory, we recommend the use of the machine manufacturer's settings, unless very unusual fluorescence measurements appear to be confounding results. If this should occur, the tests should be repeated, and if the problem persists, the machine manufacturer should be contacted for assistance.

Three replicate assessments of C_Q are obtained for each sample under assessment. Provided plate controls fall within acceptable outcomes, the range of C_Q values for each sample should be examined. We recommend the test be repeated if this range equals or exceeds 1.5, unless all three values are greater than the value of the y-intercept in the standard curve (see below). In this instance, the amount of parasite material detected is extremely low, and we do not advise repetition of the test.

The qPCR reaction can fail to produce any fluorescence signal above background (NA). If this occurs for the three replicates for a particular sample in any one channel, this should be interpreted as a 0 eggs/g value. These values should be entered directly into the outcome datasheet, without attempting to use the standard curve to calculate standardised FWEC. The positive and negative plate controls should give the operator confidence in deriving a 0 eggs/g outcome in this instance.

The qPCR outcome is more variable at very low levels of parasite eggs. Where one or two replicate C_Q values are NA these should be interpreted as 0 eggs/g and the calculation of a mean value should take place after analysis of the other replicates via comparison to the standard curve. For quality control, these values should be interpreted as $C_Q = 50$, and the same rule of not exceeding the maximum range of 1.5 C_Q units, unless all values are higher than the y-intercept, should be applied.

Where three valid, replicate C_Q values are obtained, all three are converted to a standardised FWEC value using a logarhythmic equation-derived standard curve. The equation is derived from values obtained by qPCR using a standardised set of samples; a standard curve. The standards should be analysed by qPCR in the laboratory conducting the sample analysis, and the qPCR conducted separately for each machine used to conduct qPCR tests. The standard curve analysis should be repeated each time the qPCR machine is calibrated. Sample result data should be permanently associated with the machine and the applicable version of the standard curve equation so that correct interpretation can be undertaken and so that back-tracking for problem resolution will be enabled.

The multiplex qPCR data is collected on three fluorescence detection channels. Data for each of the three genus-level tests is obtained separately in this way. This data from each channel is handled separately throughout the analysis procedure, and used in the final analysis to create an estimate of "strongylid" FWEC, by summation. Where the mean of the three replicates is 1 egg/g or less, the outcome should be interpreted as 0. In our experience, there is too much stochastic variation in the PCR reactions for infections below the level of 1 egg/g to be interpreted reliably. A provider may choose to advise that infection with the target organism was potentially present, but for normal animals husbandry purposes, this seems unnecessary.

9. Any technical qualifications

Basic laboratory and molecular biology skills are required to perform the assay.

10. Throughput and turnaround time expectations

It is expected that the results will be available within 36 hours of receipt of the samples. Once the sample is dried, the extraction process takes approximately 6 hours to purified DNA.
Throughput will be laboratory dependant however batches of 24 would allow ease of handling.

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