final report

Project code: B.AHW.0108

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Date published: June 2007

ISBN: 9781741911787

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Development of diagnostic assays for sheep nematodes based on faecal antigen detection

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

This report summarises a project designed to develop ELISA and dipstick assays to detect nematode infections in sheep by faecal analysis. Such assays would have a significant effect on the sheep industry by allowing graziers to know the level and types of worms infecting their livestock. This information would allow decisions on the timing of drug treatments and which sheep need treatment to mitigate the effects of infection. In addition, graziers would be able to use the tests to determine which of their stud sheep can maintain low parasite burdens and are suitable for breeding parasite resistant offspring. The methods used in these studies included specific antigen isolation from infected sheep faeces and from adult worm excretions and secretions (ES) collected under *in vitro* culture conditions. Antibodies were raised in rabbits, chickens, mice and guinea pigs and then tested in a range of immunological assays to determine their antigen specificity in faecal preparations and ES products, their sensitivity and specificity in ELISA and ability to discriminate infected from uninfected faeces in dipstick assays. Problems occurred mainly as a consequence of the close relationship between these parasites and thus the sharing of many protein antigens and in the contamination of adult nematode ES with sheep proteins. These problems confounded the attempt to produce specific assays for each species and caused non-specific reactions especially when testing faecal material. A specific Haemonchus antigen was isolated from faeces and adult ES and this was used to produce antibodies which together with a substrate binding reaction allowed the development of a specific Haemonchus ELISA. A dipstick was then developed to differentiate Haemonchus infected faecal solutions using a sandwich double-antibody, binding assay. The finding of sheep protein contamination in Osteratgia ES led to the development of new methods for the isolation of adult nematode antigens. Antibodies raised against the new ES were more specific though still reacted with all 3 species of nematode. This allowed the development of an ELISA and dipstick assay that could detect all three nematode species in faecal solutions. Work on Trichostrongylus resulted in a similar conclusion and thus in the final analysis two tests were developed during the project. The first detects Haemonchus infections and the second detects all three nematode infections. If combined on one dipstick they would allow a grazier to determine 1. whether his sheep have worms and 2. if those infections include a significant number of Haemonchus worms. Such information is a significant improvement on current faecal diagnostic methods and as the tests should be able to be carried out with very little training and next to the drenching race; this work has the potential to significantly change current strategies for control of nematode infections

Executive summary

This project aimed to develop new diagnostic tests to detect the three major nematode species in sheep, *Haemonchus contortus* (Barbers Pole Worm), *Ostertagia circumcincta* (Small Brown Stomach Worm) and *Trichostrongylus colubriformis* (Black Scour Worm). These tests would enable diagnosis of these infections in sheep faeces on farm and thus required detection of worm and or sheep proteins in faeces and associated with the infections. Detection of such proteins in faeces would allow the design of tests to detect worms with minimal sheep handling and sample preparation. In order for the tests to be used on farm, dipstick assays similar to the format used for pregnancy testing would be needed.

A specific protein was isolated from the faeces of sheep infected with *Haemonchus contortus* and two methods developed to detect this antigen in infected sheep faeces. Both methods produced successful laboratory assays and one method was successful in dipstick trials though the more sensitive test does require frozen faeces. An Ostertagia test was developed by subtractive techniques to find antigens specific to infection and these produced an assay that also detected the other two species, Haemonchus and Trichostrongylus. This assay is viable in both laboratory assays and dipstick forms on frozen and fresh faeces. The Trichostrongylus work developed a similar assay to that developed for Ostertagia but this laboratory test was not adaptable to dipstick format.

Thus, the first of these new diagnostic assays detects Haemonchus infections in sheep faeces and the second detects all thee major parasite species. Both tests work in ELISA and dipstick formats and can be used in the laboratory or with further development, the dipstick could be used with minimal training, on farm. Current testing has been carried out on defined infections in pen trials. Extensive field testing is now required to determine the sensitivity and specificity of these assays in the field and to develop commercial prototypes.

The use of both assays, possibly on one dipstick, will allow graziers to determine whether their sheep carry these parasites at clinical levels and whether the Barbers Pole worm is present. Barbers Pole worm is particularly dangerous as it sucks blood from its host and is among the most resistant to current chemicals. Its specific diagnosis will allow chemicals designed to target this worm to be used when needed and not wasted.

The potential of these assays is to allow graziers to determine the levels of these major worm infections in their sheep and thus only drench as required to control the infections. Such knowledge will reduce the usage of the current drenches slowing the development of drench resistance and giving better control of the production losses caused by worms every year. The use of such assays will also allow new strategic worm control programs to be developed for each farming system and the development of integrated pest management practices. Finally assays that detect these worms will enable graziers to identify sheep that do not carry significant numbers of these parasites and use these animals to breed worm resistance into their flocks. If development continues as currently planned, commercial forms of these assays may be available within 3 years.

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1 Background

Sheep nematodes present a growing problem with resistance to current drugs at high levels and few other viable methods of control available. A major problem is identifying the prevalence of nematodes in a flock and which sheep require treatment. If treatment can be limited to only those sheep that are clinically infected resistance development to current drugs would slow significantly. In addition, if a grazier could determine which sheep were not infected despite the general presence of nematodes, sheep resistant to worms could be bred and control costs could be reduced significantly. Such decisions require a cheap, rapid and on-farm diagnostic method.

2 Project objectives

To develop diagnostic tests against the three major nematode parasites of sheep.

These tests are aimed to detect nematode antigens in the faeces of infected sheep by the use of ELISA and dipstick technology.

To carry out sufficient testing of the faecal antigen tests developed in the project to allow prediction of their potential for field testing for sheep nematode infections on farm.

3 Methodology

Initial plans were to produce monoclonals against faecal antigens from each species of nematode and then use these to develop the ELISA and dipstick assays. These attempts were unsuccessful as monoclonals were not able to be produced against the Haemonchus antigen. As a result, other methods of specifically binding the major Haemonchus antigen (GST) were trialled and the GST substrate, glutathione, was used to bind and isolate the GST molecule. Anti-GST antibodies were produced and ELISA and dipstick assays developed.

Methods used for the other tests were necessarily less specific as single faecal antigens were not available. As a result, adult worm antigens were isolated as excretory secretory products and these were used as starting points for polyclonal antibody production and subtractive affinity purification before another round of antibody production. Problems were experienced with sheep protein contamination of adult worm ES preparations and this led to the use of new methods of ES isolation and further affinity purification for antibody production. Rabbits were the preferred animal for antibody production though mice were also used for low levels of protein injection and rapid testing. Chickens and Guinea pigs were trialled with limited success.

Antibodies showing preferred antigen reactivity were used to develop ELISA and dipstick tests for each nematode.

ELISAs were developed for each nematode with indirect or sandwich assays being the preferred technique as they are more easily transferred to dipstick immunochromatographic formats.

4 Results and discussion

4.1 Haemonchus contortus diagnosis

Development of the test for the Barbers Pole worm, *Haemonchus contortus*, was based on the finding by a LTU PhD student prior to this project that the protein, Glutathione S-Transferase (GST) was a possible diagnostic candidate. GST was found in sheep faeces in detectable

quantities, was apparently produced by all stages of the parasite and was excreted by the parasitic stages.

GST is able to be isolated from protein mixtures by substrate capture and this technology is well established in the literature. Initial studies under the CRC/MLA grant were directed to the establishing the best conditions for isolation of GST using the substrate glutathione and the production of antibodies that would specifically recognise the Haemonchus GST. Multiple experiments showed that very specific conditions are required to successfully and efficiently isolate GST from sheep faeces. Extensive purification of protein components before glutathione capture were not especially helpful and yields from a centifuged faecal suspension were quite adequate providing specific buffer conditions were met (Fig 1).

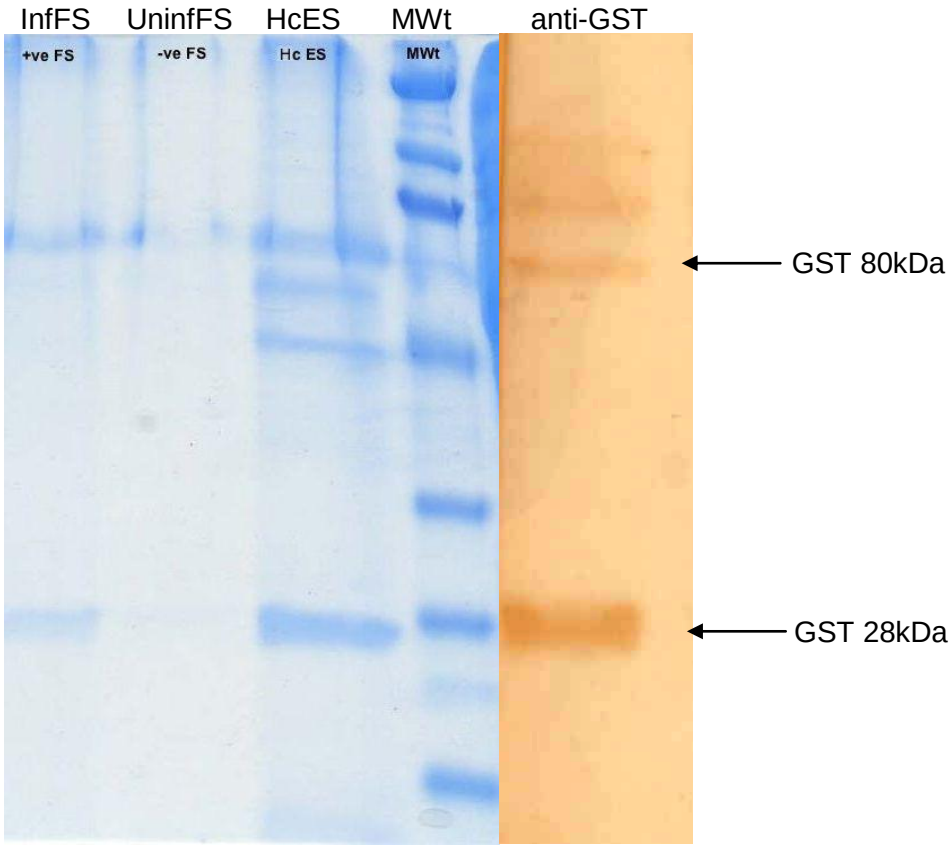

Figure 1. GST isolated from Hc ES and infected and uninfected faecal supernatants (FS) and probed with rabbit anti 28kDa GST.

However, faecal derived GST was not used for antibody production as there was a strong suggestion that this preparation would be contaminated with sheep GST (Fig 1) and possibly microbial protein. Rabbits were instead injected with GST isolated from *Haemonchus contortus* adult worms, which were taken from the sheep, washed extensively and then held in culture media for up to 24 hours (Hc ES). The media was collected, the protein component separated and this was used as a source of GST for glutathione capture (Fig 1). Injection into rabbits yielded antibody that reacted with Hc GST on immunoblots and with a molecule at similar molecular weights in faeces (Fig 1). It must also be noted that other proteins were also recognised by these rabbit anti-GST antibodies and at least one of these was also present in uninfected sheep faeces. Attempts to purify the GST further were not particularly successful until better methods of adult worm isolation and incubation were developed. As discussed later contamination of adult ES with sheep proteins has been found to be a major issue in this work.

Development of ELISA based detection of GST was trialled from the first isolation of anti GST antibodies. Initial experiments were not particularly successful though later iterations of antibody and continual improvements in the ELISA method through faecal supernatant purification, blocking solutions, secondary antibody absorption and then biotinylation of detection antibody all resulted in improvements to the assay so that it is able to be used in ELISA and dipstick tests.

However, the better ELISA format resulted from the use of glutathione capture as the first step in the assay. Initially commercial glutathione-coated plates were used to capture GST direct from faecal suspensions before detection with anti-GST antibody but we then developed our own GST coating method which improved results. This is now the preferred ELISA method for GST detection (Fig 2).

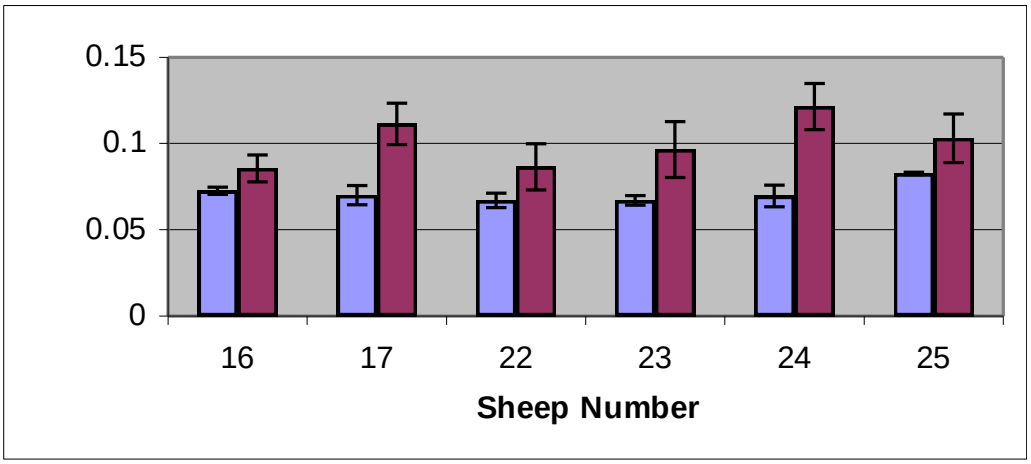

Figure 2. GST capture ELISA on FS from infected (red) and uninfected (blue) faeces

Dipstick assays which require capture of the antigen (GST) from faeces and then the binding of gold labelled antibody for detection, were less complex although the specific conditions necessary for glutathione capture has precluded this form of assay to date. Thus, the dipstick assay is at the moment dependant on antibody capture and detection (Fig 3). In ELISA format this form of assay is less sensitive than the glutathione capture assay and work is continuing on adaptation of the more sensitive assay to dipstick.

The most recent experiments have suggested that the current ELISA is more sensitive after freeze thaw of the faeces than when testing fresh faeces. This suggests that GST is released from eggs by disruption during the freeze thaw process. This finding has yet to be explored but would suggest that a method to disrupt eggs before testing may enhance sensitivity. We are also in the process of cloning the Haemonchus GST to obtain a pure source of antigen for rabbit immunisations.

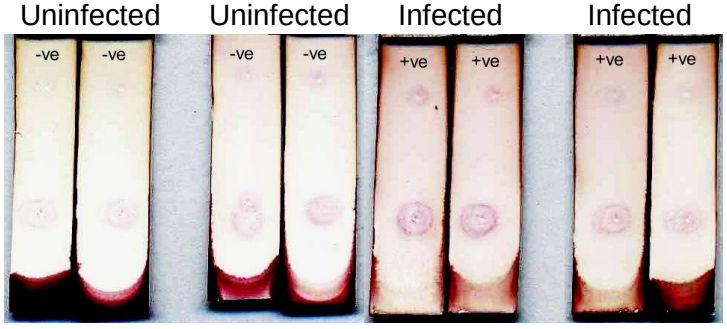

Figure 3. Haemonchus dipsticks in duplicate of two infected and two uninfected sheep.

4.2 Ostertagia circumcincta diagnosis

The work on Oc. began without the benefit of a clear antigen candidate and for this reason general antigen preparations were prepared from adult worm ES. These proteins were isolated and injected into rabbits. The antisera showed reactivity with Oc ES and faecal supernatants but stronger activity with Haemonchus ES and significant activity with negative faecal supernatants (Fig 4).

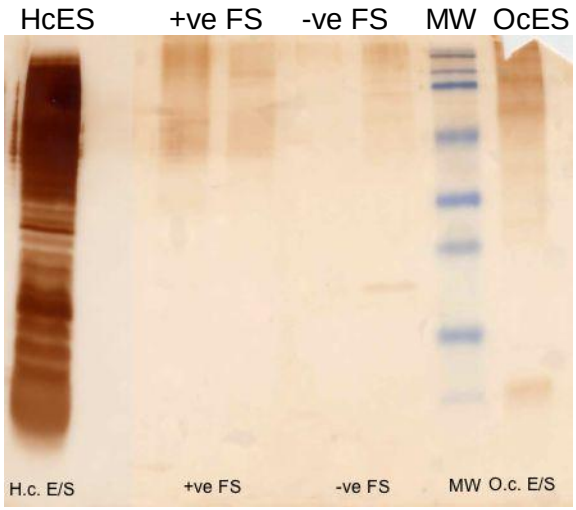

Figure 4. Immunoblot of ES and FS antigens from Hc and Oc probed with rabbit anti-Oc ES.

In order to increase specificity affinity columns were made with the anti-Oc antibody and an anti-Hc antibody. These columns were then used to absorb Oc ES proteins negatively on the Hc column and positively on the Oc column. This resulted in a new subset of proteins that were injected into another pair of rabbits. This antisera was again tested against faecal supernatants and ES preparations and showed significantly less non-specific reactivity (Fig 5). This antisera reacted well in ELISA to detect Oc infected faeces though it still showed some reactivity with Hc faeces, there was also a notable loss of activity in binding faecal proteins (Fig 6).

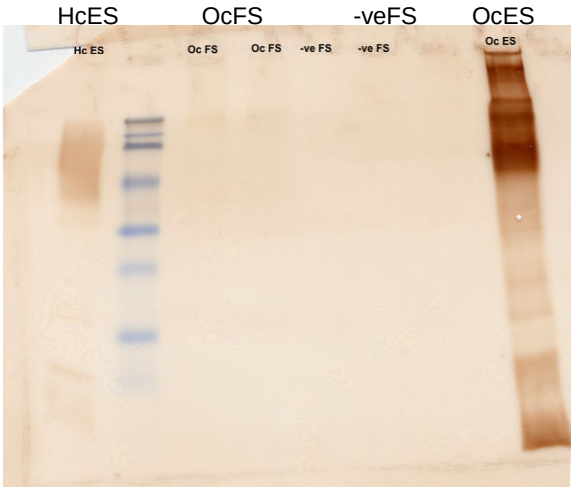

Figure 5. Immunoblot of ES and FS antigens from Hc and Oc probed with rabbit anti-Oc affinity purified antigen.

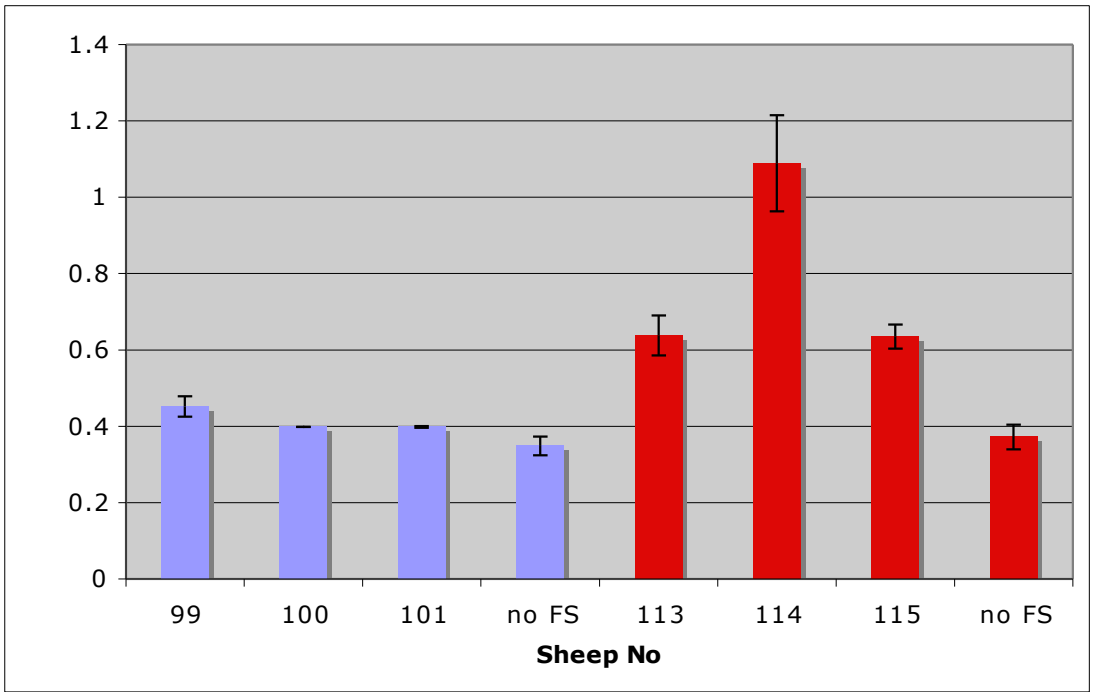

Figure 6. Anti-Oc ELISA results for infected (red) and uninfected (blue) Ostertagia faeces – sheep serum was used to absorb the detection antibody.

Further investigation of the cross reactivity of the anti-Oc antisera was conducted by absorption during the ELISA. Sheep serum was able to reduce the level of non-specific reactivity significantly (Fig 7) and this suggested that sheep protein contamination of the original ES preparation might be the issue.

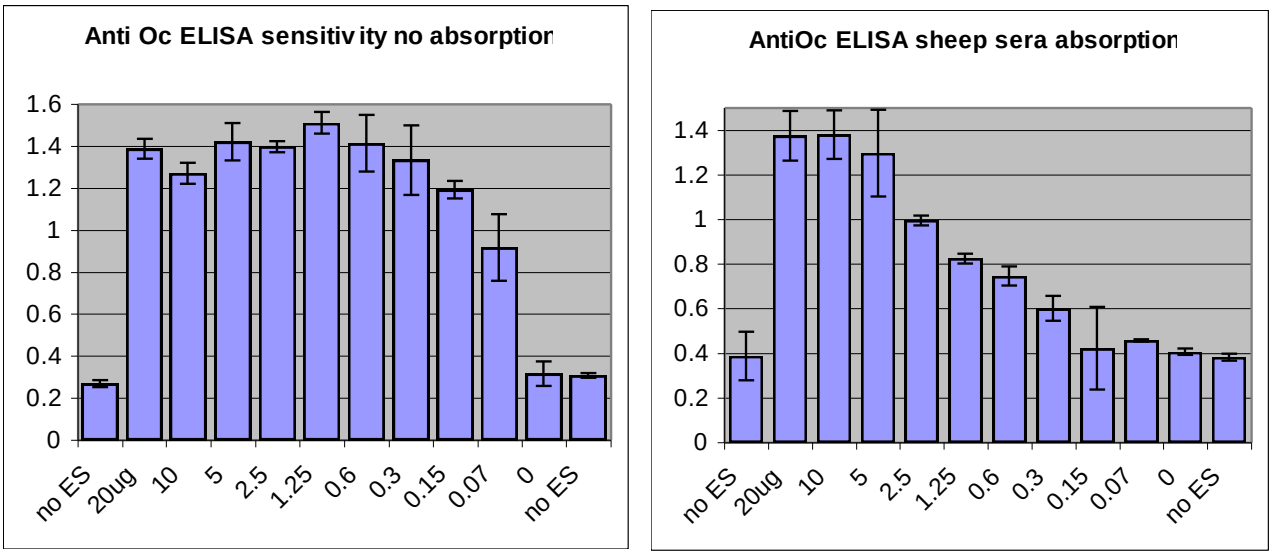

Figure 7. Oc ELISA sensitivity test of Oc ES added to FS with and without sheep sera absorption.

The suggestion that adult nematode excretory/secretory products might contain significant quantities of sheep proteins was confirmed in immunoblot assays. Figure 8 shows the range of sheep serum proteins recognised by the anti- OC ES antisera.

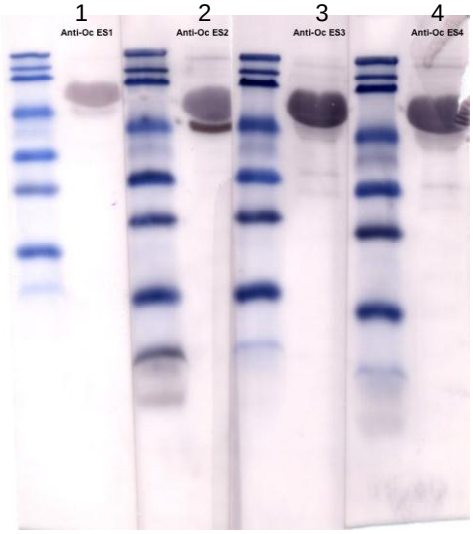

Figure 8. Sheep sera proteins probed with 4 different rabbit anti-Oc ES antisera.

These results led to a reassessment of the collection procedures for ES from adult Oc worms and a new method was developed after discussions with Paul Presidente at PIRVic. Antibodies developed against this new ES preparation showed much less non-specific activity in the ELISA (Fig 9).

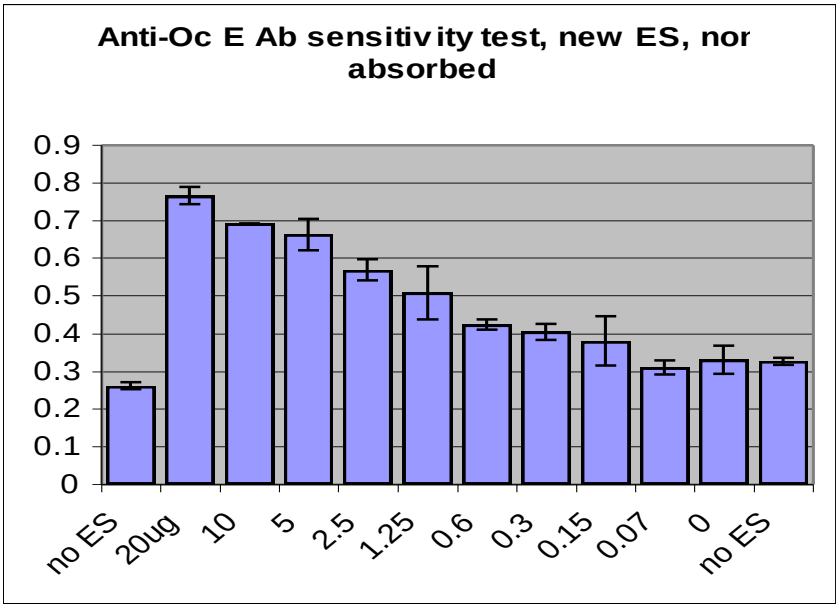

Figure 9. New Oc ES tested for sensitivity against an anti-Oc ES antisera in ELISA format, no sera absorption.

Testing of the new antisera still showed cross reactivity with Haemonchus and Trichostrongylus but much less false positive reaction. As a result this assay would be a suitable all parasite test (Fig 10).

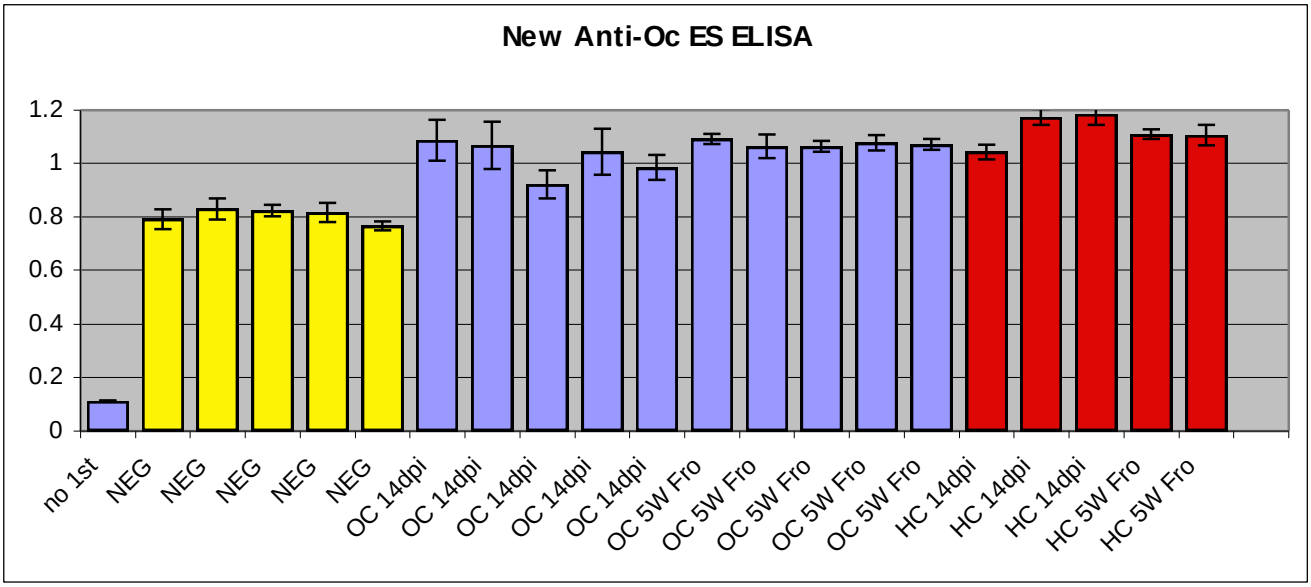

Figure 10. New Anti-Oc ES ELISA of negative, 14 day post infection fresh and 5 week post infection frozen faeces for both Ostertagia and Haemonchus infected sheep

We have also tried this test in dipstick format and the results are similarly clear cut. This assay is able to detect all three major nematode infections in sheep in either frozen or fresh faeces. The ELISA can also detect pre-patent infections at least at 14 dpi. Though, the dipstick is less sensitive at this time point (Fig 11).

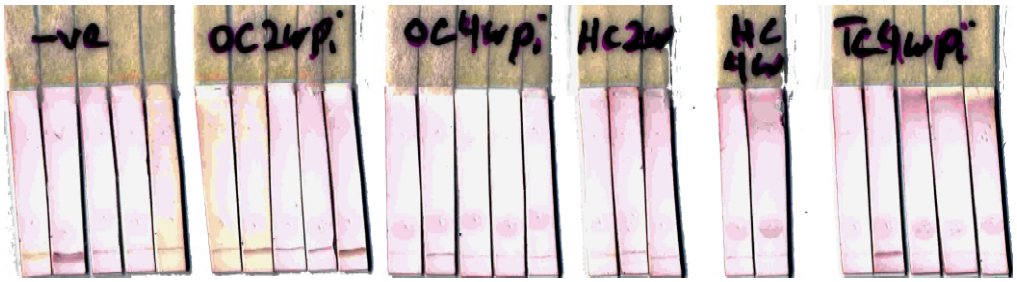

Figure 11. Dipstick assay of faecal solutions from uninfected (-ve) Oc infected at 14 and 28 days post infection, Hc infected at 14 and 28 DPI and Tc at 28 DPI. Pink dots indicate positive reactions.

The new ES preparation also allowed a repeat of our initial affinity purification protocol to purify Oc specific faecal proteins. Affinity purification of faecal supernatant on an anti-Oc new ES antibody column was followed by a Mono Q ion exchange column. Comparison with uninfected faecal supernatant isolated by the same technique revealed a number of infection specific bands most notably at about 7000 Da which is reactive with rabbit anti-ES sera(Fig 12). This protein has been injected into rabbits and will be sequenced.

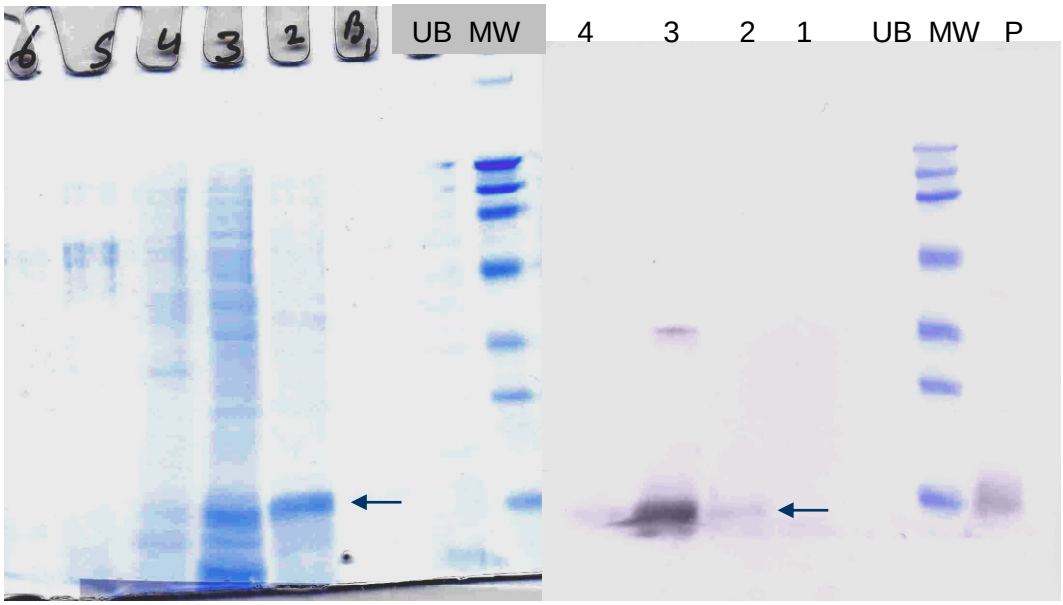

Figure 12. SDS Page and immunoblot with anti-new ES of Mono Q fractions (UB = unbound, Bound Ftns 1 to 6, P=Parent preparation) of affinity purified Oc ES (arrow = major protein of interest).

These studies have now yielded a new faecal diagnostic test that detects all three major nematode species in sheep by ELISA and Dipstick. We have also identified a major antigen in faeces that is released by Ostertagia infections but also appears to be present in Haemonchus infections. We have not yet confirmed its presence in Trichostrongylus infections. This last antigen identification step is important as it may allow IP protection of the nematode parasite assay which would be unlikely using the current procedure.

4.3 Trichostrongylus colubriformis diagnosis

The Trichostrongylus project has been largely undertaken by Steve Cotton as part of his PhD studies. There had been no previous studies of Trichostrongylus faecal proteins or the diagnostic potential of faecal testing prior to this project. As with Ostertagia the initial stages in developing a diagnostic test included isolation of adult worms and culture *in vitro* to produced excretory-secretory products (Tc ES). In addition, Steve carried out extensive studies on faecal proteins from infected sheep using a wide range of methods and analytical techniques. An early finding was the high level of mucopolysaccharides in the faecal pellet of infected sheep. This appeared characteristic of the infection and some time was spent analysing the staining characteristics of the faeces to determine if a simple dye could indicate Trichostrongylus infections. Despite some initially encouraging results especially with Alcian Blue and the PAS stain (Fig 13), analysis of larger numbers of samples through a complete infection did not produce a clear association of the staining with infection. However, a more comprehensive analysis of the many stains and dyes available may be worthwhile.

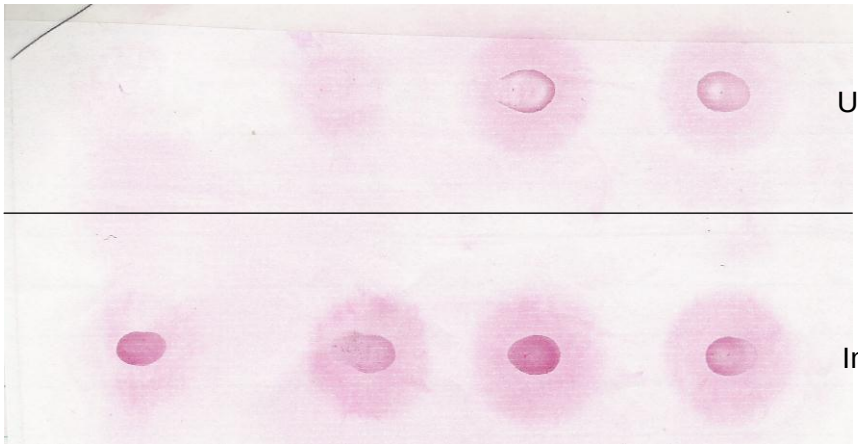

Figure 13. PAS staining of uninfected (top) and infected (bottom) sheep faecal solutions (4 different sheep per group).

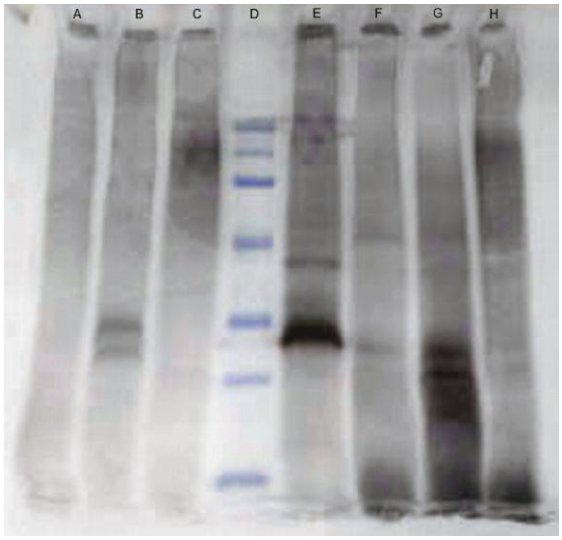

Figure 14. Reactivity of anti Tc ES antisera with faecal protein from uninfected (lanes A, B & C) and infected sheep (lanes E, F, G & H).

Antisera raised against Tc ES reacted with large range of proteins in infected and uninfected faeces (Fig 14) and showed extensive cross reactivity against both Haemonchus and Ostertagia.

As a result a similar strategy to that used for Oc was used to isolate a more purified Tc protein fraction by subtractive affinity chromatography. Tc ES was passed through an anti-Hc antibody column to adsorb Hc reactive proteins then the unbound eluate was passed through an anti-Tc column to positively adsorb the Tc proteins (Fig 15). These were injected into a number of mice to check the resultant antibodies before further test development.

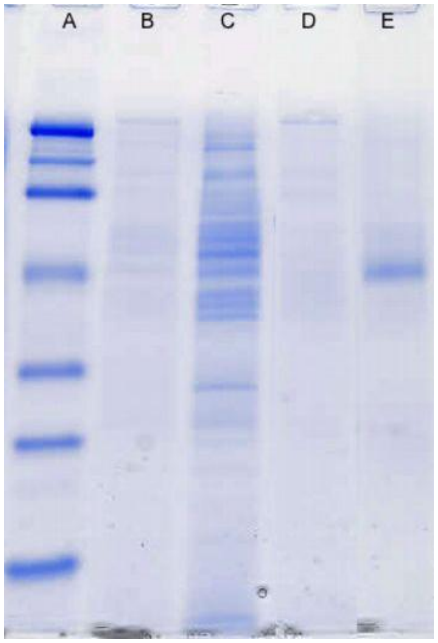

Figure 15. SDS-Page of affinity purified proteins from infected (lanes C & D) and uninfected (lanes B and E) sheep faeces after Hc and Tc affinity columns.

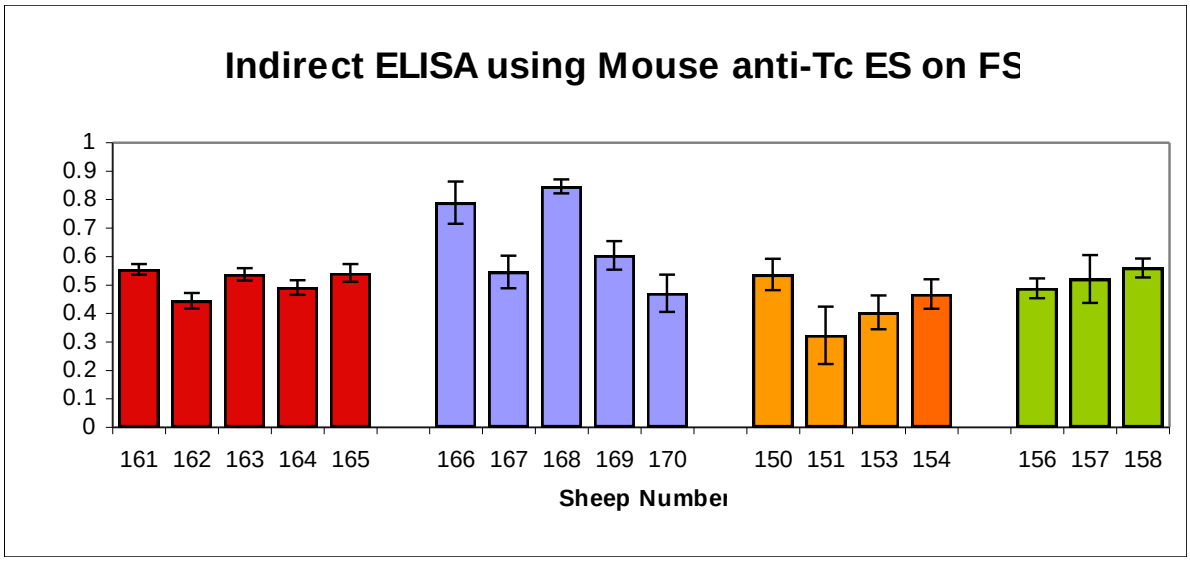

Figure 16. Indirect ELISA With faecal solutions (FS) from uninfected (red), Tc infected (blue), Hc infected (orange) and Oc infected (green) sheep.

The mouse antisera showed better reactivity with Trichostrongylus infected faecal solutions but indirect ELISA was still problematic with false negatives now the major issue (Fig 16).

The finding that Ostertagia and Haemonchus adult ES were contaminated with sheep proteins also led to a similar assessment and finding in Trichostrongylus ES. Absorption of Tc ELISA showed similar effects to those seen with the other ELISAs. However, one of the original anti Tc ES antibodies does detect differences between infected and uninfected sheep faeces that are removed by absorption with sheep sera suggesting that this antibody is recognising sheep proteins associated with the pathological reaction to this infection and these might be used to detect the infection. Unfortunately, the assay used to detect these proteins is direct and thus difficult to adapt to dipstick assays where a specific capture molecule is required.

The mouse antisera developed through the affinity purification method was more successful in direct and indirect assays though was still cross reactive with Ostertagia (Fig 17).This suggest that as in the Ostertagia test development, such methods can be used to develop ELISA diagnostic antibodies but that absorption is required with at least all the other species (Haemonchus and Ostertagia in this case). In order to develop a specific Trichostrongylus assay we would need another round of affinity purification with ES prepared by the new methods followed by antibody production and then antigen identification as has been accomplished with Ostertagia. Obviously we are of the view that such work is not warranted as the Ostertagia assay which detects all three species of nematodes when combined with a specific Haemonchus assay would be a significant improvement on existing diagnostic techniques and allow all farmers to make control decisions knowing the important parasite burdens in their flocks. We do not believe that specific knowledge of Ostertagia or Trichostrongylus infections would give any significant improvement in control over the assays already developed in this project

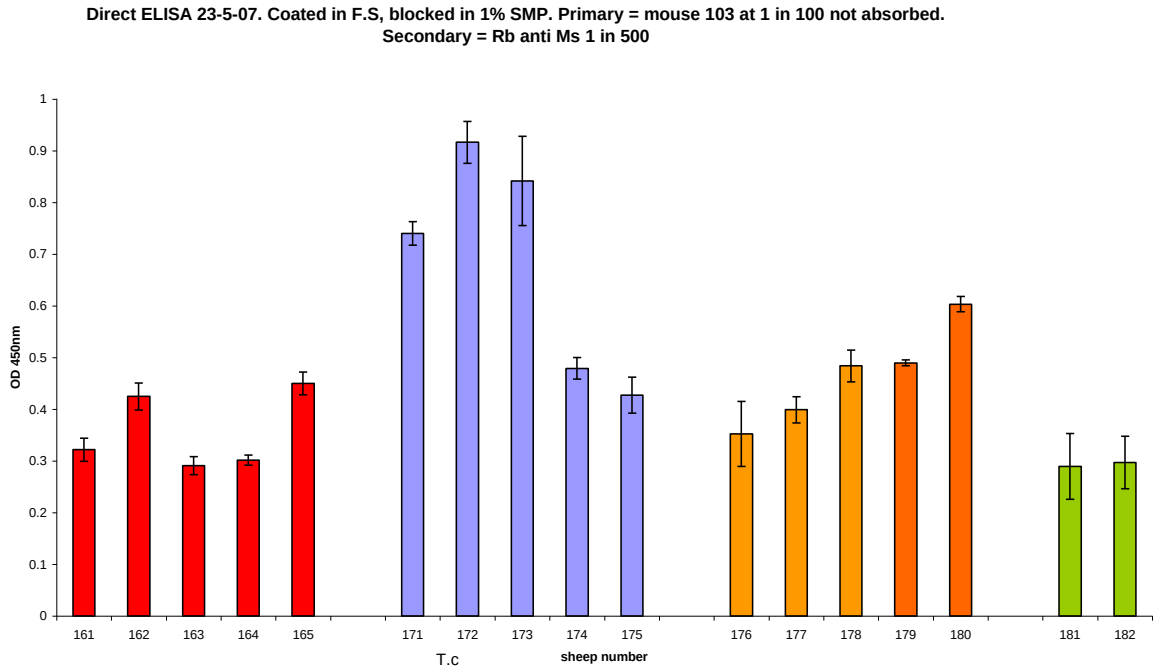

Figure 17. Mouse anti Tc affinity purified antibody in a direct ELISA showing reactivity with Tc infected sheep faeces (blue) and some cross reactivity with Oc (orange), red are negative controls and green is Haemonchus.

5 Success in achieving objectives

This project has resulted in the production of a new test for *Haemonchus contortus* and a new assay for gastrointestinal nematodes in sheep. The Haemonchus assay has been tested in both ELISA and dip-stick format and is successful in laboratory scale experiments. It is more sensitive using frozen faecal samples than fresh samples. We actually have two different forms of this assay in ELISA but only one adapted to dipsticks.

The second assay was a result of attempts to develop specific tests for *Trichostrongylus colubriformis* and *Ostertagia circumcincta*. The assay finally developed uses an antibody directed against Ostertagia ES proteins but it detects all three species of nematode tested in these trials. This test has been mainly used in ELISA format but a limited number of dipstick trials suggest that this format is also viable.

Production of these assays (ELISA or dipstick) is now feasible in numbers suitable for field testing though stability testing of the assay under various storage conditions will be necessary. Field testing will be necessary to determine the actual sensitivity and specificity of the assays and thus their usefulness in parasite testing. Commercial scale production will require the production of larger amounts of the specific antibodies and work to this end is already underway.

We believe that we have achieved the major aims of this project and are ready to proceed to field testing for validation of the assays.

The question of IP protection will be addressed in the recommendations.

6 Impact on meat and livestock industry – Now and in five years time

The development of cheap and easy to use tests for parasite infections in sheep will significantly improve the control of these infections on farms. This will occur through;

1. better diagnosis and thus more targeted treatments,
2. by only treating the sheep that are clinically infected and
3. through the use of these tests to breed only those animals that carry low burdens of parasites.

Current diagnosis is based on an assessment of clinical symptoms (the wormy lamb) and faecal egg counts. The former leaves diagnosis until it is too late and deaths are imminent, while the latter are increasingly expensive and slow if preformed off-farm and require expensive equipment and technical expertise if carried out on-farm. Dipstick assays would tell a grazier that his sheep have worms at a clinical level for around $2 per test, in less than 10 minutes with minimal training. These assays will significantly increase management options and change the usage of valuable drugs by reducing the number of treatments and allowing rational treatment decisions based on the presence of clinically important infections rather than timed treatments and dead sheep.

At this stage of development and with continuing funding dipstick assays should be available for field testing later this year. Commercial development would follow successful field testing and dipsticks could be being used on-farm in commercial evaluation studies in 2009. The quantities used in such studies could be manufactured at La Trobe University. The final commercial form would depend on these analyses and the manufacturer but commercial dipstick could be available by the end of 2009 early 2010.

In 5 years the assay should be well established in the market and we would envisage one dipstick test to simultaneously diagnose general nematode infections and Haemonchus specifically. Thus, a 3 line stick would have I line to check the assay is working, 1 line for the presence of nematodes and 1 for the presence of Haemonchus. The level of worms detected and thus the cut off point of reactivity will depend on field testing but a colour chart might be needed to differentiate different levels of infection by colour comparison with each line. Graziers would take a single pellet of sheep faeces add it to a vial with a buffer solution, break it up then place the dipstick in the solution, colour development should be complete in 10 minutes. Graziers would check a number of sheep in each mob or possibly larger numbers if breeding decisions are required.

The use of the tests might allow the development of new drenching plans and IPM solutions to slow the development of drug resistance and ensure higher productivity with reductions in the costs of control.

7 Conclusions and recommendations

This project has succeeded in developing two new diagnostic tests that for the first time could allow the detection of internal parasite burdens in sheep by the analysis of faeces on the farm. The tests have been analysed in both ELISA and dipstick format with the latter allowing easy on-farm use and results within 10-15 minutes of obtaining the faeces. Testing to date has been on animal house infections in sheep with no infections other than the defined parasite species under study. Control sheep have been kept absolutely clear of any helminth infections. As a result additional testing is now required in naturally infected sheep to determine actual sensitivity and specificity data. This should be done in cooperation with DPI and CSIRO field stations around Australia to check the tests under different environmental conditions and farming systems.

Although the original aim was to develop three tests, one for each species of nematode under study, we are confident that a test that can detect and differentiate Haemonchus infections from other nematode infections would allow defined control and breeding programs to reduce drug usage and thus resistance development. We are also of the view that the assays developed in this project can be combined into a single dipstick assay that would report infection with nematodes and/or with Haemonchus.

At this stage we would recommend that work continue on developing formats of the dipstick assays that are robust and repeatable under field conditions and that these then be trialled in experimental field situations. These field tests would necessitate faecal egg count analysis of natural or experimental infections and post-mortem of targeted animals to check worm number and species composition. In concert with these assays work on the final form of a commercial assay would be underway to enable rapid distribution of a commercial prototype. IP protection of the assay(s) and identification of suitable manufacturers would also be carried out, though we note that the production of enough dipsticks for the Australian market could be undertaken by a spin off company or similar start up with very little capitol expenditure. In fact, initial testing and ground proofing could be carried out with tests manufactured at La Tribe University using current facilities.

The IP position of the current assays requires detailed analysis but the Haemonchus GST test is almost certainly patentable and especially in its substrate capture format. The general parasite test may not be able to be protected in its general anti-ES format as similar assays have been reported in the literature. The reactivity with all the major nematode species may be novel but a more certain path is the identification of a single antigen as is currently underway. Identification of this protein and confirmation that it is effective in the assay would allow patent application.