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Evaluating methods for conducting faecal egg count reduction tests in sheep using McMaster technique, Mini-FLOTAC and pooled counts.

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Executive Summary

Worms are a threat to the long-term viability of the Australian sheep industries due to their effect on productivity, animal welfare and profitability. Overall estimate for the economic impact of worms is A$435m per year, making them the costliest disease condition affecting Australian sheep producers. The problem of worms is compounded by the grave situation of anthelmintic resistance, with 95% of farms tested showing resistance to one or more of the major drench classes used in sheep. Rapid, cheap and accurate diagnosis of worm burdens in sheep is required for monitoring sheep for treatment, detecting drench resistance and for genetic selection.

There are 29 laboratories known to provide worm egg count services to Australian sheep producers, but many others operate, including producers who are self-trained or with minimal training doing their own testing. Most are thought to use the McMaster method, which has limitations in time, cost and sensitivity. Other test methods include Mini-FLOTAC, Para-Sight, FECPAK G2, Wisconsin and total worm counts. The most practical method, particularly for individual counts, is McMaster, due to its ease of use and minimal equipment required, but it has low sensitivity, typically 50 eggs per gram.

This means that animals to be tested for genetic selection or drench tests need to be left until their worm burdens are high enough to cause adverse effects on their health and welfare. Mini-FLOTAC has the same level of practicality for pooled tests and is ten times more sensitive, raising the possibility of testing on sheep with much lower worm egg counts.

Species differentiation of worm eggs is essential for both effective management of worms and to diagnose drench resistance. Current methods used in Australian laboratories are (i) culture and morphological differentiation of larvae and (ii) PCR. Morphological techniques are cheap but slow and require highly-trained staff, while PCR is rapid and accurate but more expensive and inconvenient.

The Mini-FLOTAC method is cheap and rapid and because it has high sensitivity of 5 eggs per gram (epg) it can be used differently to current methods. There is potential for it to be used for pooled worm egg counts for monitoring worm populations on mobs of sheep, with higher accuracy than current pooled methods. New techniques for data analysis have been developed that will allow Mini-FLOTAC to be used for faecal egg count reduction tests (FECRT) with less expense, time and discomfort to animals than current methods but similar or higher levels of accuracy.

In this study, the Mini-FLOTAC method was compared to McMaster worm egg counts and the results showed a very high correlation coefficient. Subsequently, 51 Faecal Egg Count Reduction Tests were conducted on farms across Australia. From this dataset, 46 trials included both Day 0 (D0) and Day 14 (D14) untreated control (UTC) groups for comparison of methods. Four different calculations were used for comparison:

1. Conventional McMaster count at 50 epg sensitivity on 15 individuals with D14 UTC
2. Conventional McMaster count at 50 epg sensitivity on 15 individuals with D0 (pre-treatment) control
3. Mini-FLOTAC at 5 epg sensitivity with samples from 15 individuals pooled into 3 composite samples, D14 UTC
4. Mini-FLOTAC at 5 epg sensitivity with samples from 15 individuals pooled into 3 composite samples, D0 control.

Comparison of efficacy estimates from 1171 combinations of product x worm species showed that there was very little overall difference between the four methods. However, using untreated animals in a control group led to problems with variation from the Day 0 worm egg count and potential morbidity due to high worm burdens. These problems were exacerbated when the level of *Haemonchus* was high in the mob (>30%). Because of this, estimates of efficacy using Day 0 controls are deemed to be more accurate and were associated with lower risk of morbidity, particularly in flocks with high level of *Haemonchus*.

Efficacy calculations from all of the 51 completed FECRTs were collated and trends in sheep worm drench resistance were determined. The 2 new products Zolvix (monepantel) and Startect (derquantel and abamectin in combination) performed with an average efficacy greater than 95% in the 3 main worm species. All other single active ingredient products tested had efficacy lower than 95%, the exceptions being levamisole against *Haemonchus* (99.7%) and the macrocyclic lactone products abamectin and moxidectin against *Trichostrongylus* (96 and 97% respectively).

Key messages are:

1. Worm egg counts using Mini-FLOTAC have a high correlation with counts conducted using McMaster.
2. Pooled Mini-FLOTAC counts can be reliably substituted for individual McMaster counts in laboratories conducting faecal egg count reduction tests to determine anthelmintic efficacy.
3. Worm egg count results from untreated animals on Day 0 can be used instead of Day 14 untreated control values to determine drench efficacy.
4. Using Day 0 controls avoids problems associated with untreated control groups, particularly in flocks with *Haemonchus* levels higher than 30%.
5. Extension to farmers and their advisors on drench testing and efficacy will lead to better health and welfare for sheep flocks and improved productivity and profitability for sheep producers.

Introduction

Gastrointestinal worms are present on all livestock farms in Australia and grazing animals are exposed to worm larval contamination in their paddocks. Infections with internal parasites pose a high economic burden, estimated at A$435m/year, to the Australian sheep industries (Lane et at., 2015). Most of those costs are related to loss of production, with the remainder to the purchase and application of drenches. If drenches have low efficacy, the money is spent without achieving any benefits.

Effective worm control benefits sheep producers by improving lamb birth weights (Johnstone et al., 1979), milk yield of ewes (Fthenakis et al., 2005), time to turn-off of lambs (Altaif et al., 1979), weight and body condition score of ewes (Darvill et al., 1978), wool production (Callinan & Thomson, 1981), carcass composition (Arsenos et al., 2007; Coop et al., 1982), weaning weight (Macchi et al., 2001; Anderson 1972), and subsequently weaner survival, particularly in Merinos (Campbell et al., 2009).

A meta-analysis published in 2016 (Mavrot et al., 2015) estimated that mixed infections due to poor control of worms in sheep flocks led, on average, to reduced:

- wool growth –by 10%
- milk yield - by 22%.
- weight gain –by 26% (adjusted figure 15%).

The assessment of liveweight to determine productivity costs probably underestimates the effect of worms, due to the fact that even low worm burdens significantly increase the weight of the gastrointestinal tract in affected sheep (Jacobson et al., 2009).

Apart from the production benefits mentioned above, flow-on benefits of good worm control in sheep flocks include a lower risk of flystrike, reduced diarrhoea and wool staining, improved ease of shearing and crutching, as well as lower rates of morbidity and mortality (Roeber et al., 2013; Sutherland & Scott, 2010). Despite advances to educate producers on integrated pest management (Kahn & Woodgate, 2012; Bailey et al., 2009), both in Australia and overseas (Miller et al., 2011), control of worms on sheep properties still largely relies on effective anthelmintics (drenches).

However, resistance to anthelmintics is a major and increasing problem (Besier & Love, 2003). A recent survey conducted in Australia showed that 95% of Australian sheep farms tested had resistance to one or more of the major drench groups (Playford et al., 2014).

Sustainable worm management involves FEC monitoring and regular drench resistance tests (Sayers & Sweeney, 2005). However, many Australian sheep producers fail to conduct FECs or FECRTs (LeFevre, 2013).

This is possibly due to:

a) the perception that these are costly
b) the time-consuming nature of current tests
c) drench tests are often not possible due to low starting FECs in sheep (LeFevre, 2013)
d) lack of producer awareness or education (Woodgate & Love, 2012).

Current standards stipulate a starting FEC of 300-400 eggs per gram average in mobs being tested (Hutchison, 2009), although there is a suggestion that this could be revised to 150 epg (Coles et al., 2006). More sensitive FEC methods would justify using lower starting (or control) FECs (Levecke et al., 2012) and make it possible to conduct more drench resistance tests than currently possible.

In this study, drench efficacy tests were performed using two different protocols:

- The standard approach used in Australia based on the traditional McMaster method (sensitivity of 50) of 15 head of sheep per group counted individually, including an untreated control group, and

- A recently established new FECRT protocol which uses a Day0 control rather than an untreated control group, facilitating a more realistic/true efficacy calculation, while at the same time enabling the treatment of all animals at the same time without the risk of debilitating health due to untreated progressing worm infections. This protocol also utilises the relatively new Mini-FLOTAC method which provides a higher sensitivity in combination with pooling the faecal samples, which reduces the overall costs of a more sensitive method.

The results obtained by the two protocols as well as the costs and the impact on animal welfare were compared to formulate future recommendations.

Current methods of determining worm burden include the McMaster method, PCR, FLOTAC, Para-Sight, FECPAK G2, modified Wisconsin method and post-mortem total worm count. Since its description in 1941, the McMaster technique has been widely employed. The techniques for sampling and processing of samples for conducting a FECRT have been internationally standardised (Coles et al. 1992) and the design of the McMaster counting chamber provides consistent sample volume, allowing extrapolation of number of eggs counted per gram of faeces using a simple calculation.

The main disadvantages of this technique include the potential for overestimation of egg numbers (Cringoli et al. 2004) and low analytic sensitivity of about 50 epg (Hutchinson 2009, Torgerson, Paul & Furrer 2014, Bosco et al. 2014). The Mini-FLOTAC employs similar preparation techniques to the McMaster method, however the design of the thermoplastic device allows the separation of floating eggs from debris in the solution, resulting in a clearer field of view for egg counting (Ianniello et al. 2015). Although the technique requires more time than the McMaster method, it allows significantly greater analytic sensitivity, down to 5 epg (Rinaldi et al. 2011, Godber et al. 2015).

Both methods require staff training and laboratory equipment, including flotation solution, accurate scales, measuring equipment and microscopes. In both cases, the Faecal Egg Count (FEC) is calculated by dividing the product of number of eggs counted and the dilution factor by the weight of the sample in grams:

$$EPG = \frac{eggs\ counted\ \times dilution\ \ factor}{sample\ \ \ weight}$$

FEC methods with high sensitivity are beneficial in informing management decisions. Sensitive methods, such as the Mini-FLOTAC method, can detect light worm infestations that would be missed by less sensitive tests such as the McMaster method. Prior to introduction, new diagnostic methods must be sufficiently sensitive, accurate, convenient and cost effective for both the provider and the consumer. New tests should ideally be validated against both existing tests and the gold standard and have high reliability.

Project Objectives

The key aim of this project is to assess a novel method for conducting faecal egg count reduction tests (FECRTs) by comparison with three other methods.

Specific Project Objectives were:

1. Explain the project to state-based local organisers to recruit interested farms.
2. Perform screening (pre) tests to include a total of 50 farms.
3. Conduct FECRTs on the recruited 50 farms, following a protocol using:
    a. McMaster technique at 50 eggs per gram sensitivity using 15 individual counts compared against an untreated control at Day 0 (day of treatment).
    b. McMaster technique at 50 eggs per gram sensitivity using 15 individual counts compared against an untreated control at Day 14 (14 days after treatment).
    c. the Mini-FLOTAC method at 10 eggs per gram sensitivity, pooling 15 samples into 3 composites, comparing against the Day 0 untreated control group.

4. The Mini-FLOTAC method at 10 eggs per gram sensitivity, pooling 15 samples into 3 composites, comparing against the Day 14 untreated control group. Perform statistical analysis to determine the efficacy of each tested drug on each individual farm. (as well as an overview of general performance of different drug classes).
5. Formulate key messages following important findings regarding the anthelmintic efficacy situation.
6. Promote project outcomes and benefits more broadly to wool growers though industry communication channels.

Note that the funding for this project covered laboratory testing and analysis, while all fieldwork including recruiting farms, conducting drench trials, submitting samples and other related activities was covered by AWI extension agents and other stakeholders on a voluntary basis.

Methodology

Comparison of the McMaster and Mini-FLOTAC:

For this part of the project, thirty randomly chosen ovine faecal samples from Worm Test Kits processed at Dawbuts Pty Ltd for FEC and/or larval culture and differentiation between 28 March 2017 and 12 April 2017 were used.

Each Worm Test Kit contains three rows of five sample cells. From the samples, 2g faeces were collected for the McMaster test. For the Mini-FLOTAC method, 5g of the contents of the cells were pooled into 3 groups, corresponding to the rows; this gave a total of 90 pooled samples. Prior to removal of faeces from the cells, the content of each cell was thoroughly mixed to ensure homogenous egg distribution. The sample weight was determined using A&D HT-120 electronic scales which were calibrated at the beginning of the day and tared prior to measuring each sample. For details of the methods see sections below.

The statistical analysis was performed in Excel. The sum of the number of strongyle eggs for each sample was obtained. This number was multiplied by 50 to obtain total eggs per gram (epg) for the McMaster test and by five for the Mini-FLOTAC. The FECs calculated using McMaster and Mini-FLOTAC were compared for each sample by finding the difference between the FECs.

The level of agreement for the two tests was estimated using the absolute difference in FECs between McMaster and Mini-FLOTAC for each sample. The levels of agreement were defined as very high (≤ 50 epg difference), high (51-100 epg difference), medium (101-150 epg difference), low (151-200 epg difference) and very low (>200 epg difference).

The mean FEC was then plotted against the difference in FECs between the tests for each sample; the FEC as reported by the McMaster technique was also plotted against the FEC reported by Mini-FLOTAC for each sample. A concordance correlation coefficient was determined to compare the paired samples (Doohoo, Martin & Stryhn 2003, pp. 87-91).

Acquisition and inclusion of participants:

Media press releases, personal contacts, state veterinarians and AWI contacts were used to acquire participants for this study. While the costs for the laboratory performance of the tests were completely subsidised by the sponsor, farmers needed to provide their own drenches, which were specified in the protocol as per the respective Animal Ethics Committee (AEC) approval (for details regarding the drenches see FECRTs).

Pre-tests were performed and the mean FEC had to be over 250 epg for the property to be included in the study.

Conduct of faecal egg counts:

McMaster:

For the modified McMaster method, individual samples were used. This method involved placing each 2g pooled faecal sample into a clean 60 mL glass container and macerating with a small volume of tap water until a soft paste was formed. Saturated saline solution was added and the sample thoroughly mixed. A plastic aeration tube connected to an OTTO Air Pump SA-800 was placed into the container to agitate and homogenise the solution; during this time, a loading syringe was used to aspirate some of the solution and load 0.5 mL into 1 McMaster slide chamber. This process was repeated for the remaining samples. Each McMaster slide chamber was then viewed systematically at 4x magnification using a Prism Optical stereoscopic microscope, resulting in a sensitivity of 50 epg.

Mini-FLOTAC:

For the Mini-FLOTAC method, pooled samples were used. The 15 individual samples per group were pooled in three pools, each containing five samples, and those pooled samples were thoroughly homogenised.

The modified Mini-FLOTAC method involved placing 5g of faeces into a clean 60 ml glass container and macerating it with a small volume of tap water until a soft paste was formed. Saturated saline solution was added and the sample thoroughly mixed. A plastic aeration tube connected to an OTTO Air Pump SA-800 was placed into the container to agitate and homogenise the solution. A loading syringe was used to aspirate some of the solution and fill the two chambers of a Mini-FLOTAC chamber were filled per sample and eggs counted systematically at 4x magnification using a Prism Optical stereoscopic microscope, resulting in a sensitivity of 5 epg.

Additionally, a group-based faecal culture was established to determine the parasite genera present before and after treatment.

On the day of treatment, the 15 faecal samples for the Day 0 control were combined, mixed with vermiculite and incubated for seven days at 27 degrees. This allows the development from eggs through to third stage larvae, which can be used to determine the genus (e.g. *Haemonchus*, *Trichostrongylus*, etc.) and sometimes also the species (e.g. *H. contortus*, *H. placei*, *Tr. colubriformis*, *Tr. axei* etc.). This provides information regarding the composition of the parasite population present before treatment.

On Day 14, group-based cultures were performed as described above for every treatment group as well as the untreated control. This provides information on the efficacy of each treatment against all parasites or, if not, which genus/species survived the treatment. It also provides information on how the parasite population composition of the untreated control group changed in comparison to the Day 0 control.

Faecal Egg Count Reduction Tests (FECRTs):

For the FECRTs, initially five single drenches (with only one active ingredient) were chosen, containing the following actives: Closantel (CLO), Moxidectin (MOX), Monepantel (MPL), Levamisole (LEV) and a Benzimidazole (BZ). Six months later two additional drenches were added: Abamectin (ABA) and Startect® (DER), which contains Abamectin and Derquantel. The inclusion of the additional drenches was not mandatory, leading to a different number of data sets available per drench.

On the day of treatment (Day 0), faecal samples were taken from 15 sheep (or picked up from the ground if freshly deposited) to be used as the Day 0 control. Then sheep were allocated to groups of 15 and each group treated with a different drench. For the standard protocol, one group was left untreated to serve as the untreated control group (UTC).

All sheep were identified to the respective treatment group using eartags, zip ties or spray raddle. Identification method was determined by each individual producer according to their preferences.

A second set of samples were taken 14 days later. A faecal sample was collected from every individual sheep (in total 15 samples per treatment group).

Efficacy calculation:

The initial calculation of efficacies was performed in the ResoLoot spreadsheet (Microsoft Excel, modified by Robert Dobson) in order to be able to provide timely feedback to the participating farmers. Results emailed back to the farmers only included those obtained by following the standard protocol using the UTC and the McMaster method.

Statistical analysis of the different methods and protocols:

For the calculation of the efficacy as well as comparison of the different methods/approaches, data was entered into an Excel spreadsheet. The data was then analysed by specialist veterinary statistician Dr. Ahmad Rabiee.

The following analyses were performed:

1. Standard protocol including the untreated control group using 15 individual samples and the McMaster method (sensitivity 50 epg) **=> Method 1**
2. New protocol utilising the Day 0 control group using 15 individual samples and the McMaster method (sensitivity 50 epg) **=> Method 2**
3. Standard protocol including the untreated control group using 3 pools of samples and the Mini-FLOTAC method (sensitivity 5 epg) **=> Method 3**
4. New protocol utilising the Day0 control group using 3 pools of samples and the Mini-FLOTAC method (sensitivity 5 epg) **=> Method 4.**

Results

Comparison of the McMaster and Mini-FLOTAC methods

For the initial comparison of Mini-FLOTAC and McMaster, 90 ovine faecal samples from 30 mobs were tested. The level of agreement between the FEC results from McMaster and Mini-FLOTAC was very high in 41.1 % of samples, high in 26.7 % of samples, moderate in 7.8 % samples, low in 5.6 % samples and very low in 18.9 % samples. When sorted by mean sample FEC, the percentage of samples with each level of agreement changed.

The percentage of each level of agreement for FEC between McMaster and Mini-FLOTAC results were divided into lots of 250 epg. There were 54 samples with mean FEC ≤250, 19 samples with mean FEC 250-500 epg, 5 samples with mean FEC 501-750, 1 sample with FEC 751-1000 and 11 samples with mean FEC >1000. As the mean FEC in the samples increased, the level of agreement between the tests tended to drop, with Mini-FLOTAC reporting a higher FEC than McMaster (Figure 1; level of significance not tested).

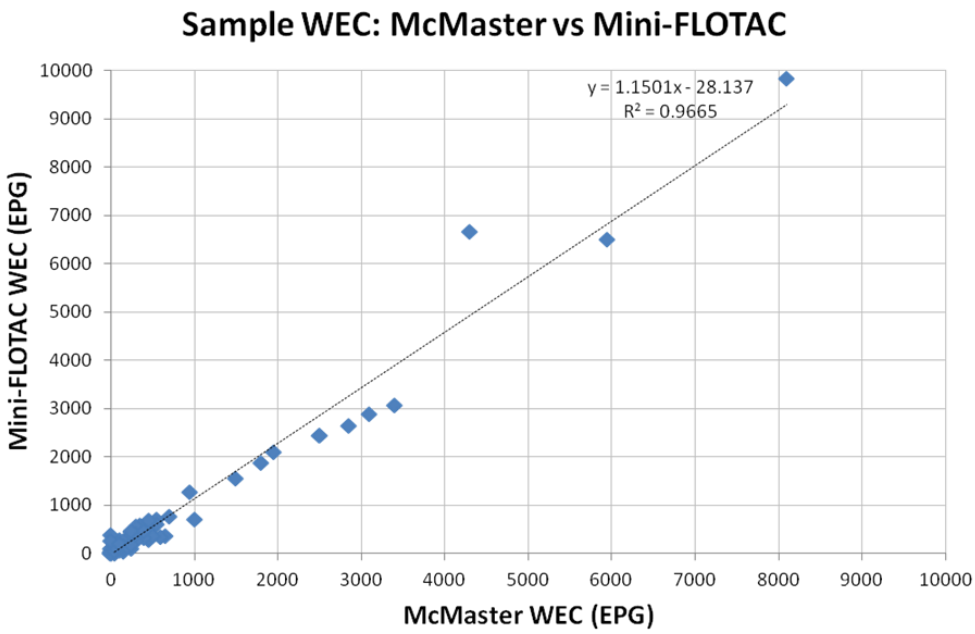

Figure 1: FEC for samples by both McMaster and Mini-FLOTAC with linear trendline.

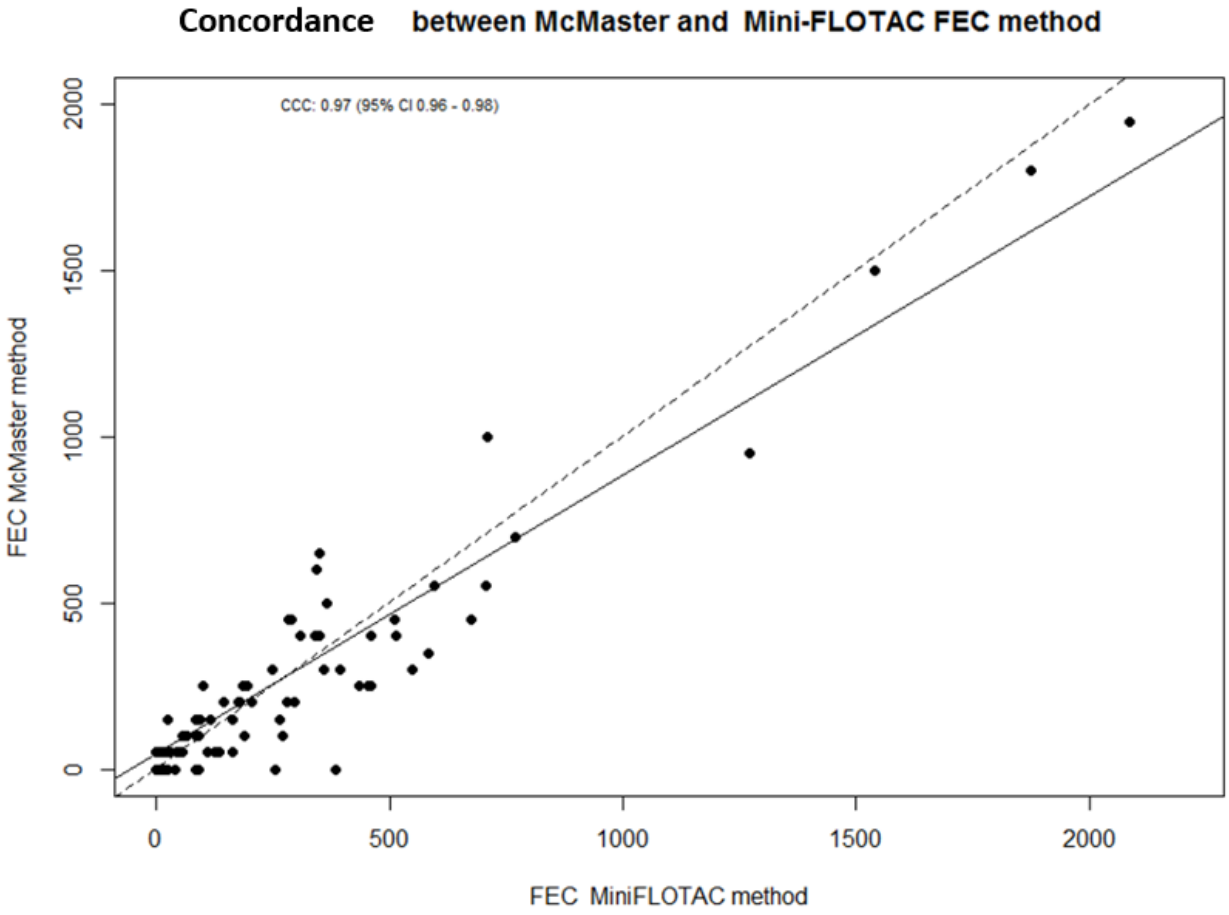

Figure 2: Concordance between FEC from McMaster and Mini-FLOTAC methods.

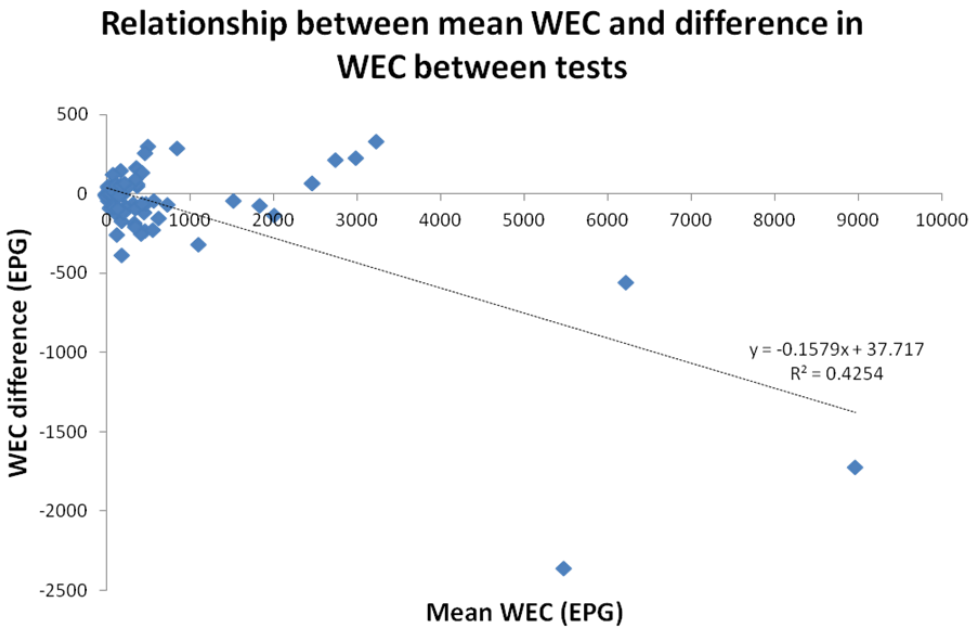

Figure 3: Difference between FEC reported by McMaster and Mini-FLOTAC techniques, with linear trendline. A difference in FEC less than 0 occurred when FEC reported was higher for Mini-FLOTAC than McMaster.

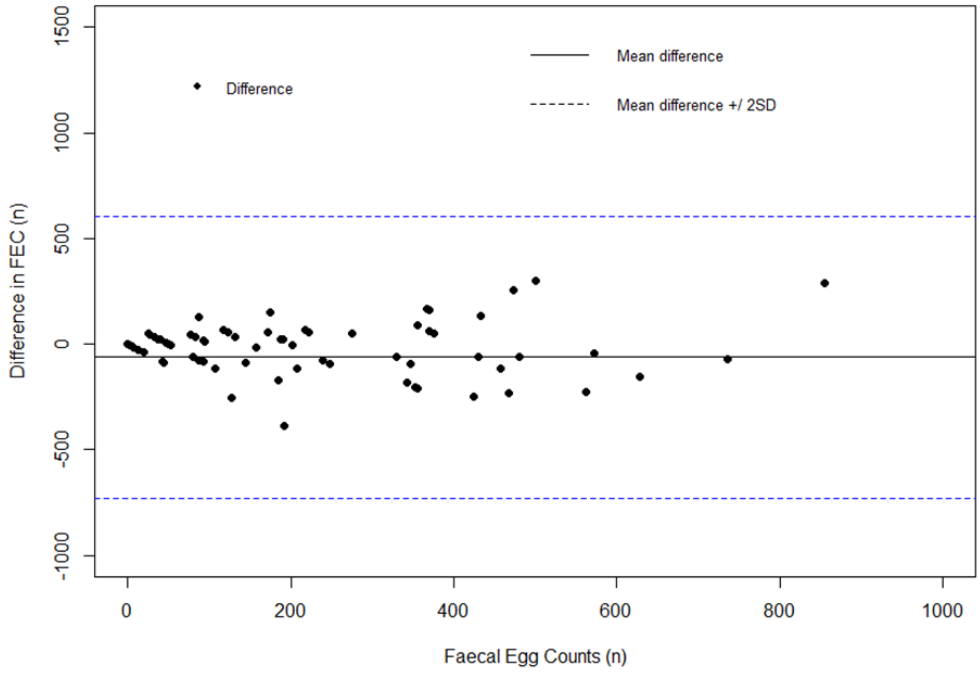

Figure 4: Difference in FEC (epg) between McMaster and Mini-FLOTAC techniques, by mean FEC. The concordance correlation coefficient was calculated to be 0.97 (95 % CI 0.96-0.98).

The mean loading time was 1 minute, 10 seconds for the McMaster chambers and 4 minutes, 14 seconds for the Mini-FLOTAC devices. Similarly, the mean counting time was 4.3 times longer for the Mini-FLOTAC technique than for the McMaster technique. The mean time required to count 10 eggs was 9 minutes, 6 seconds for McMaster and 7 minutes, 52 seconds for Mini-FLOTAC; while the mean time required to count to 100 EPG was 1 minute, 49 seconds for McMaster and 15 minutes, 44 seconds for Mini-FLOTAC.

There was a difference in FEC of ≤ 100 in 67.8 % of the samples tested and a difference of greater than 150 epg in just under a quarter of the samples, and there was a concordance correlation coefficient of 0.97 (95 % CI 0.96-0.98) between the results of the two tests. The magnitude of the difference in FEC between tests tended to be positively correlated with mean sample FEC.

Acquisition and inclusion of participants:

Potential co-operators were contacted by local extension agents working in the state-based networks, rural retail stores, veterinarians and pharmaceutical company representatives. Drought conditions throughout 2017-2019 across much of Australia led to low worm burdens in sheep and consequently low FECs in the screening tests (see Figure 5 below).

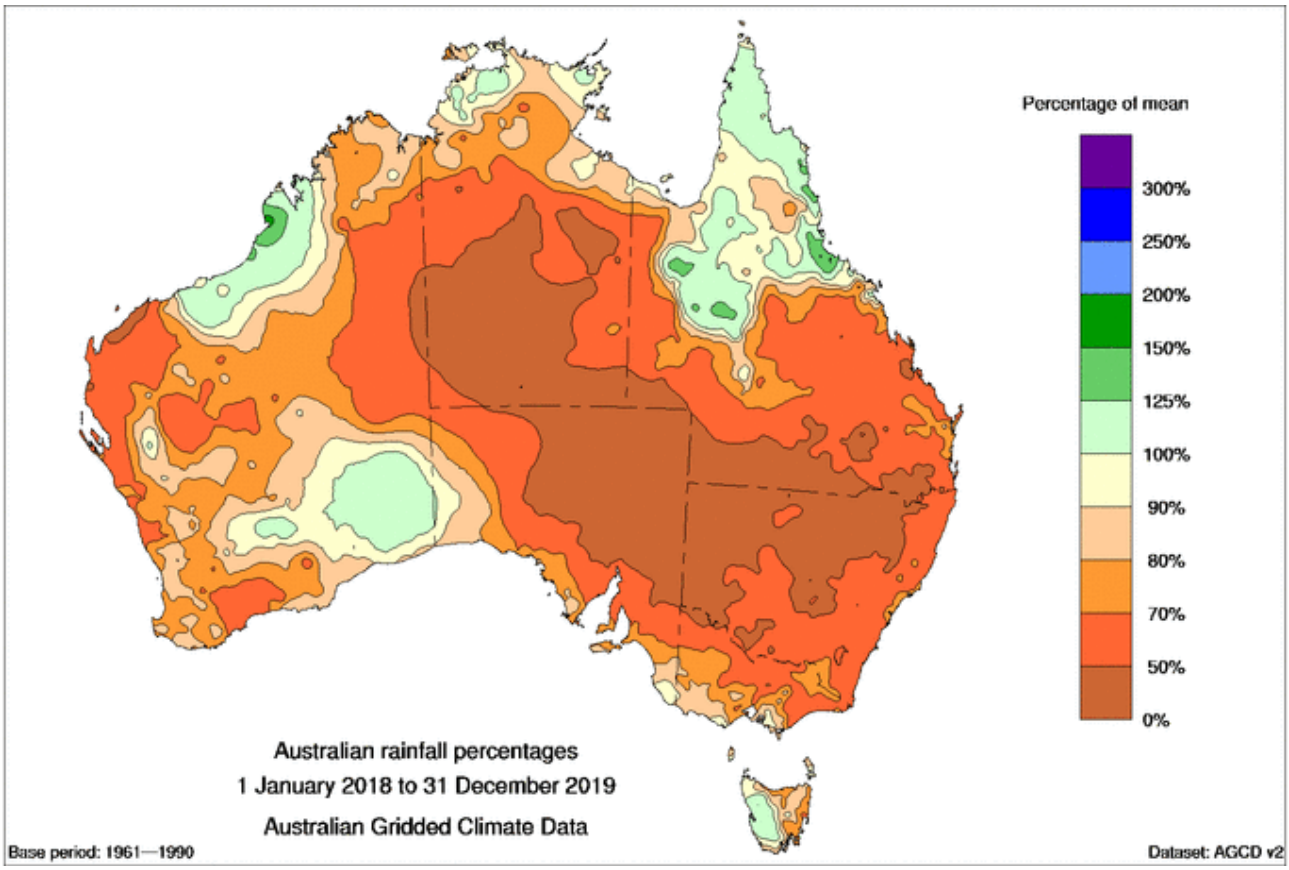

Figure 5: Rainfall as a percentage of historical averages across Australia in the period 2018-2019. Source: Bureau of Meteorology).

A total of 74 worm egg count screening tests were conducted across five states (see Table 1 below). Of these, 46 completed FECRTs with sufficient starting worm egg count (150epg) to be included in the resistance survey. FECRT method comparison was conducted on 51 and 49 farm results for Day 0 and untreated control (UTC) respectively.

Victorian flocks were under-represented, with only 4 screening tests and one completed drench test. The remaining successful tests were apportioned across states relative to sheep populations, with the highest number of both screening tests and completed drench tests (27 and 19 respectively) in NSW (see Figure 6). No tests were conducted in Queensland due to the inability to secure the necessary research permits and animal ethics approvals.

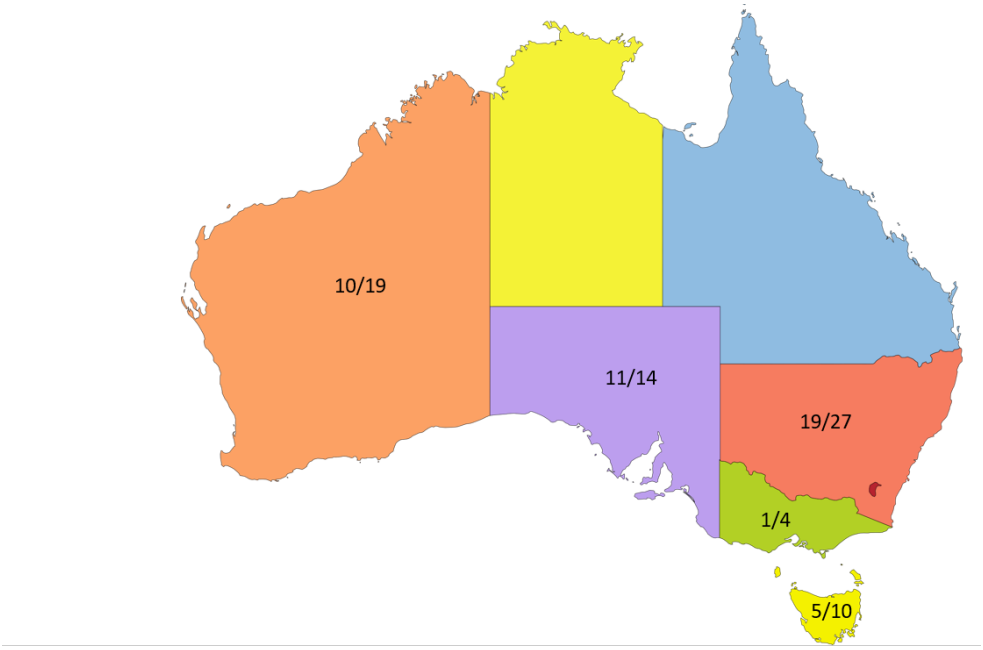

Figure 6: Locations of screening tests (second numeral) and successfully completed full drench resistance trials (first numeral) included in this study.

Faecal Egg Count Reduction Tests (FECRTs):

Seventy-four farms were included in this study. However, untreated control groups and Day 0 control samples were not implemented on every farm and not all farms achieved the requirements for resistance data analysis.

In total, 49 FECRT results were used to compare the results obtained by Mini-FLOTAC and McMaster for the protocol including the UTC. For the Day 0 group protocol, 51 FECRTs were available. For analysis of drench resistance, a minimum worm egg count of 150 epg (Day 0 or Day 14 using McMaster) was established, with 46 sites achieving this requirement.

State	Sheep population (2019 MLA figures)	% of national flock	Screening tests	Drench efficacy analysis and comparison	% of total tests
NSW	25.2m	35%	27	19	41%
Victoria	14.7m	21%	4	1	2%
SA	11.8m	17%	14	11	24%
Tasmania	2.2m	3%	10	5	11%
WA	14.5m	20%	19	10	22%
TOTAL	71m		74	46	

Table 1: Location and number of farms participating in the drench resistance trials compared to the distribution of the national sheep flock.

Control worm egg counts

The worm egg counts on Day 0 (day of treatment) averaged 808 eggs per gram (epg), while those of untreated control sheep on Day 14 (14 days post-treatment) averaged 794 epg, a difference of only 14 epg, or 1.68% of the Day 0 value (p=0.95). However, analysis showed that although the difference between averages was only small, due to the difference between D0 and D14 going up and down, the absolute difference between D0 and D14 values was high, at 432epg or 54% of the D0 mean epg value.

Comparison of Day 0 and Day 14 untreated control worm egg counts (n=46) showed reasonable correlation, (R2 = 0.6527), between the two counts (see Figure 7). The entire set of untreated control values and differences is shown in Appendix 1.

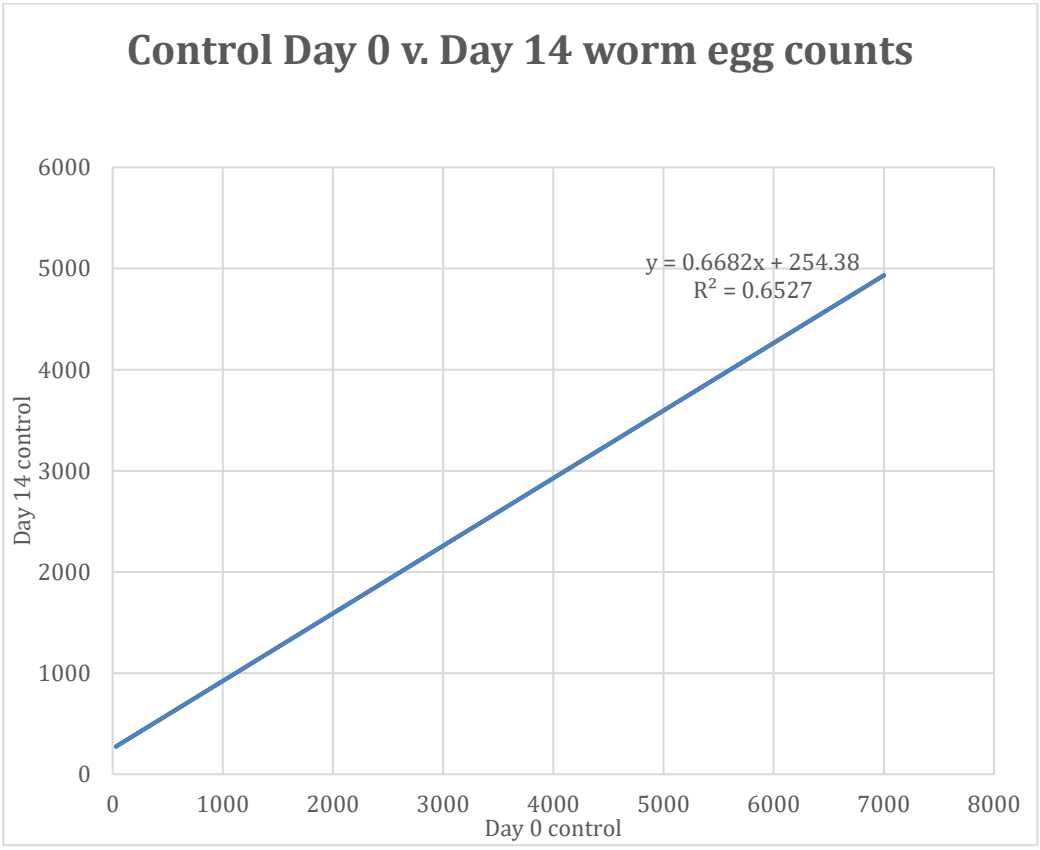

Figure 7: Correlation between Day 0 and Day 14 control worm egg counts on 46 sheep farms across Australia.

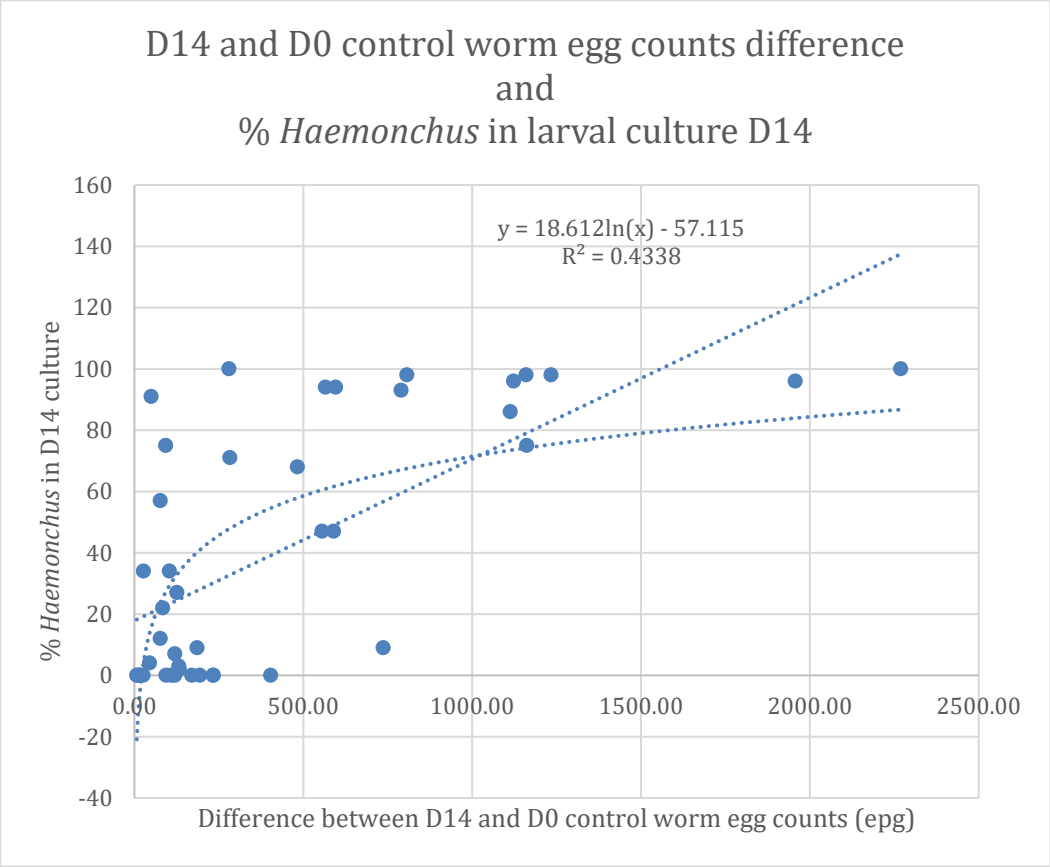

Figure 8: Difference between Day 0 and Day 14 control worm egg counts plotted against % *Haemonchus* in the D14 larval culture.

On average, *Haemonchus* made up 41% of the eggs cultured from the Day 0 samples, with a range of 0-100%. The difference between Day 0 and Day 14 control worm egg counts was higher when *Haemonchus* made up a greater percentage of the Day 14 larval culture. The mean difference between D0 and D14 untreated control counts when *Haemonchus* was less than 30% (n=22) was 149 epg, while the mean difference on farms when *Haemonchus* was greater than 30% (n=21) was 730 epg (see Figure 9 below). A one-way ANOVA analysis conducted using Microsoft Excel showed the difference is significant at p=0.0001.

The greatest D0-D14 untreated control difference observed was 2269 epg, on a farm with 100% *Haemonchus* cultured from the D0 sample. The greatest D0-D14 untreated control difference observed on a farm with less than 30% *Haemonchus* was 737 epg, when 9% *Haemonchus* was recovered from the D0 culture.

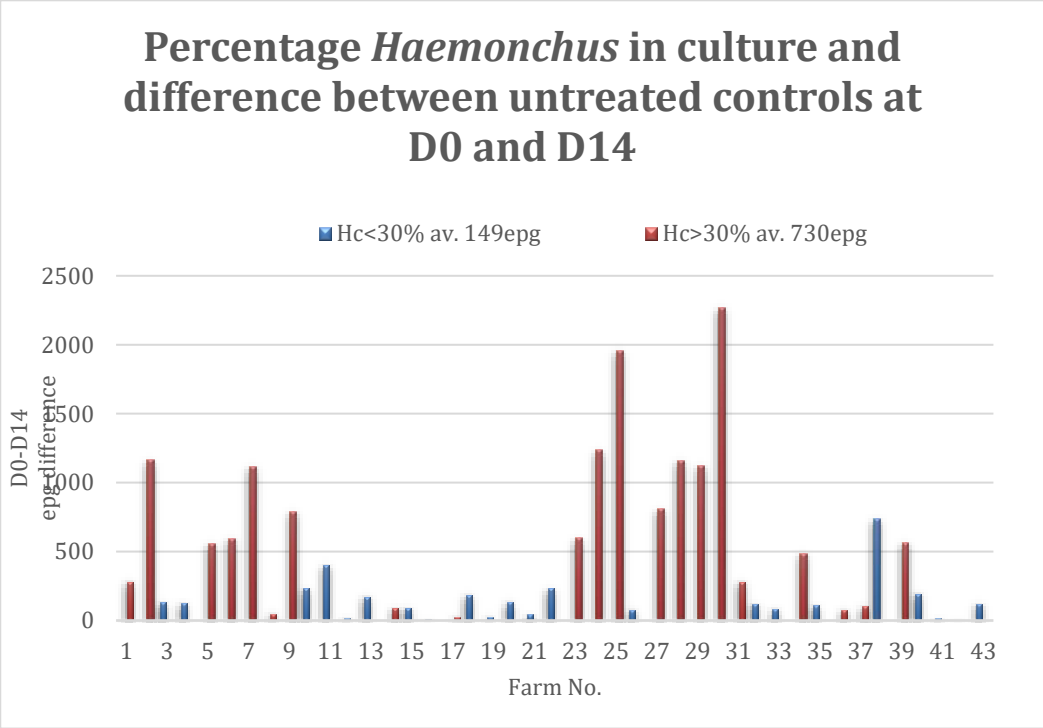

Figure 9: Difference between D0 and D14 worm egg counts shown for each farm, with percentage *Haemonchus* in D0 culture shown as blue (<30%) or orange (>30%). Average difference of 22 farms with Hc<30% was 149epg, while average difference of 21 farms with Hc>30% was 730epg, p=0.0001.

Active ingredients tested

The trial protocol was devised for ease of implementation across multiple users on farms across the country. Based on advice from the ParaBoss Technical Committee, it was decided to examine the efficacy of single active ingredients, including just one representative of the macrocyclic lactone/milbemycin class, moxidectin.

Products containing single active levamisole, benzimidazole (albendazole, fenbendazole or oxfendazole), closantel and monepantel were also included. Due to individual preference of co-operators, as well as variation in resistance and worm species prevalent across the country, different active ingredients were added or included in some of the drench tests. Table 2 below shows the active ingredients and combinations used and the number of trials.

Active ingredient	No. of trials
Monepantel	45
Benzimidazole	45
Levamisole	45
Moxidectin	44
Closantel	35
Abamectin	33
Derquantel + abamectin	17
Moxidectin + benzimidazole + levamisole	3
Naphthalophos + benzimidazole + abamectin	1
Abamectin + levamisole + benzimidazole	1

Table 2: Active ingredients and combination products used in this study across Australia.

Combination product efficacy

The efficacy of combination products such as abamectin+levamisole+benzimidazole can be estimated from the efficacy of the individual components, using the WormBoss combination drench efficacy calculator, available online at www.wormboss.com.au/sheep-goats/tests-tools/management-tools/drenches/combination-drench-efficacy-calculator.php.

Number of tests and efficacy against different genera of nematodes based on Method 1

Efficacy using the traditional standard method (Method 1- 15 individual counts using McMaster slides at 50epg sensitivity and comparison against the Day 14 untreated control), was reported to participants during the trial. Table 3 below shows a summary of the number of trials and efficacy for the various active ingredients against the 3 main worm genera. Note that not all 3 genera were detected in all locations. The number of trials where each genus is detected is listed below.

Efficacies of various active ingredients and combinations are shown in Table 3.

New products

The average efficacy of monepantel against all species is 99.8%, while against *Teladorsagia* it is 99%. The lowest efficacy recorded for monepantel against *Trichostrongylus* was 80% and against *Teladorsagia* was 86%, both on the one farm. The control worm egg count on this farm was 1027 epg, with 8% *Trichostrongylus* (82 epg) and 1% *Teladorsagia* (10epg). The reliability of the efficacy estimate for *Trichostrongylus,* (80%, with upper and lower 95% confidence intervals of 29 to 94), is reasonably robust. However, the estimate for efficacy of *Teladorsagia* in this trial (86% with upper and lower 95% confidence intervals of 50-96) is not based on sufficient numbers of eggs observed in the controls and has lower reliability.

Monepantel efficacy against *Teladorsagia* was reported as 92% in one other trial. In all other drench trials in this study (n=43) monepantel had efficacy greater than 95% for all genera.

Derquantel + abamectin (Startect) was included in 17 trials, with average efficacy against all species of 99.7% and average efficacy of 99.4% against *Teladorsagia*. The lowest recorded efficacy was 96% (CI 59-100) against *Teladorsagia*.

Older products

In contrast to the new products (monepantel and derquantel + abamectin), the older active ingredients all had overall average efficacy of less than the recommended standard of 95% (see details in Table 3 below).

All active ingredients had efficacy of 100% against at least one species of worm on at least one farm, indicating that worms susceptible to all of the currently-used active ingredients still exist in some areas.

Efficacy of the single active products against the 3 main genera of sheep worms was variable and mainly below the required level for effective worm control. The exceptions are levamisole against *Haemonchus*, where all of the 33 tests had efficacy greater than 95% and the two macrocyclic lactones, moxidectin and abamectin, which both had all but one test (25/26 for abamectin, 38/39 for moxidectin) showing greater than 95% efficacy against *Trichostrongylus*. In this study, average efficacy of abamectin against *Trichostrongylus* was 96%, while moxidectin's average was 97.3% across Australia.

Efficacy	**All species**	***Haemonchus***	***Trichostrongylus***	***Teladorsagia***
Derquantel + Abamectin				
Number	17	11	14	16
Av efficacy	99.6%	99.9%	99.6%	99.4%
Low	98%	99%	97%	96%

High	100%	100%	100%	100%
No. >95%	17	11	14	16
tests >95%	100%	100%	100%	100%
Monepantel				
Number	46	33	40	43
Av efficacy	99.6%	99.8%	99.4%	99.3%
Low	97%	97%	80%	86%
High	100%	100%	100%	100%
No. >95%	46	33	40	43
tests >95%	100%	100%	98%	95%
Benzimidazole				
Number	46	33	39	43
Av efficacy	69.5%	75.7%	76.8%	51.7%
Low	0%	0%	0%	0%
High	100%	100%	100%	100%
No. >95%	4	11	13	5
tests >95%	9%	33%	33%	12%
Abamectin				
Number	33	24	26	30
Av efficacy	71.5%	58.0%	96.0%	88.4%
Low	0%	0%	0%	0%
High	100%	100%	100%	100%
No. >95%	15	7	25	16
tests >95%	45%	29%	96%	53%
Levamisole				
Number	46	33	39	43
Av efficacy	90.7%	99.7%	86.1%	76.7%
Low	20%	97%	0%	0%
High	100%	100%	100%	100%
No. >95%	24	33	22	7
tests >95%	52%	100%	56%	16%
Moxidectin				
Number	45	32	39	43
Av efficacy	84.3%	78.4%	97.3%	87.1%
Low	0%	0%	0%	0%
High	100%	100%	100%	100%
No. >95%	22	17	38	23
tests >95%	49%	53%	97%	53%
Closantel				
Number	36	32		
Av efficacy	57.8%	92.1%		
Low	0%	42%		
High	100%	100%		
No. >95%	5	22		
tests >95%	14%	69%		

Table 3: Efficacy of different active ingredients against the 3 main genera of sheep nematodes as well as overall efficacy using Method 1. Note that Closantel is a narrow-spectrum drench targeting *Haemonchus*.

Comparison of worm egg count methods

Method 1 vs Method 2:

These two methods are both based on using the McMaster FEC method, performed on 15 individual samples. The farmers received the results generated following the standard protocol including an UTC (Method 1). When comparing these results to the ones obtained by Method 2, significant differences in efficacies were revealed. For those farms which had predominantly *Haemonchus* present before treatment, the FECR calculated was significantly lower when using a Day 0 control. This is due to the fact that there was an average difference between D0 and D14 untreated control counts of 730 epg on farms with >30% *Haemonchus*.

In this situation, using a Day 0 control provides a more accurate representation of drench efficacy, compared to using an Untreated Control Group, as explained using the following example:

Scenario A

On Property X, the pre-treatment FEC averaged 500 epg and the parasites present were 85% *Haemonchus*, 10% *Teladorsagia* and 5% *Trichostrongylus*. After treatment, the treatment group had a FEC of 200 epg. However, the untreated control group suffered from the increasing *Haemonchus* infection and is now displaying a FEC of 5,000 epg and 100% *Haemonchus*. If the post-treatment FEC of 200 epg is compared to those diseased animals, the drench efficacy seems to be good (around 96%). But those treated animals had a FEC of 500 epg before the treatment and now still have a FEC of 200 epg, which means the drench was in reality only 60% effective. Comparing the results between the treated and untreated control group to determine efficacy is in this case misleading.

Scenario B

On farms with low levels of *Haemonchus* the situation is different. For example, on Farm Y, the Day 0 control FEC is 400 epg with only *Teladorsagia* and *Trichostrongylus* present. The untreated control group FEC 14 days later is still around the 400 epg mark, because both of those parasites are not high egg-producing parasites. Therefore, a true efficacy calculation is more likely.

Method 1 vs Method 3:

The results were obtained using the same protocol but different egg counting method were again found to be different. This was particularly noticeable for tests performed on farms with low FECs present before treatment. Here, the better sensitivity of the Mini-FLOTAC method comes into effect, revealing some samples still positive after treatment, whereas the McMaster method failed to pick up the egg counts below 50 epg and classifies those samples as negative.

Regardless of the value of the pre-treatment FEC, a negative FEC after treatment leads always to the assumption of full efficacy of the tested active. However, when compared to (even low) FECs still present after treatment, the efficacies calculated are lower. This difference was not observed for farms with a pre-treatment FEC above 2,000 epg, where the increased sensitivity of the Mini-FLOTAC method is not an important factor.

It also has to be noted that the statistical comparison of the data obtained for the same group by the two different methods revealed that the pooling into 3 pools (each containing five samples) can offset the rigour of the efficacy estimates.

Comparison of the 4 different FEC methods used:

The comparisons were between:

a) McMaster method with UTC vs McMaster method with Day 0
b) Mini-FLOTAC method with UTC vs Mini-FLOTAC method with Day 0
c) McMaster method with UTC vs Mini-FLOTAC method with UTC
d) McMaster method with Day0 vs Mini-FLOTAC method with Day 0

Comparisons a and b allow to assess differences due to using a different approach while maintaining the same FEC method (for both methods, McMaster & Mini-FLOTAC). In comparisons c and d the protocol remained the same while the different FEC methods were compared against each other.

Two different modelling approaches were used:

1. Ordinal Logistic Regression (OLR) (with McMaster/Day0 as a reference group)
    a. Model 1 FEC ~ protocol
    b. Model 2 FEC ~ protocol + FarmID
2. General Estimating Equations (GEE) (same reference group)
    a. Model 1 FEC ~ protocol + FarmID.

Methods/Approach	**N**	**Mean ± SD**	**Range**
McMaster-UTC	295	78.00 ± 30.70	(0.00, 100)
McMaster-Day0	283	76.10 ± 32.40	(0.00, 100)
MF-UTC	303	76.7 ± 30.50	(0.00, 100)
MF-Day0	290	78.2 ± 32.30	(0.00, 100)

Table 4: Descriptive statistics showing means of efficacy estimates almost equivalent across the 4 methods.

Efficacies for all drugs used in all four scenarios (McMaster UTC, McMaster Day0, Mini-FLOTAC UTC and Mini-FLOTAC Day0) were calculated as described above using the ResoLoot spreadsheet. Mean efficacies including the 95% confidence intervals were then used for further comparison.

Methods were compared by assigning efficacy results to 4 categories (below).

1. =0%
2. >0% & ≤50%
3. >50% & ≤95%
4. >95%

Methods/Protocols	**Efficacy (%)**				**Total**
	0.0%	> 0% ≤ 50%	> 50% ≤ 95%	> 95%	
McM-UTC	22 (7.0%)	22 (7.0%)	114 (39%)	137 (46%)	295
McM-Day0	21 (7.0%)	34 (12%)	104 (37%)	124 (44%)	283
MF-UTC	21 (7.0%)	27 (9.0%)	119 (39%)	136 (45%)	303
MF-Day0	26 (9.0%)	29 (10.0%	107 (37%)	128 (44%)	290
	90	112	444	525	1171

Table 5. Proportion of efficacy by Methods/Protocols

As can be seen in Table 6. the estimate of the number of tests with zero efficacy was the same (7%) for the first three methods, while rising to 9% using Method 4. At the other end of the scale, estimates for acceptable efficacy (>95%) was also almost equivalent across methods, varying only from 44-46% over 525 tests.

Estimates of efficacy in the ranges 0-50% (9-12% of tests) and 50-95% (37-39% of tests) were also in a similar range.

Methods & approaches	No	Coefficient (slope)	95%CI	Wald test	P value
McMaster-UTC *vs.* McMaster-Day 0	578	-1.93	(-7.36, 3.50)	0.486	0.486
MF-UTC *vs.* MF-Day 0	593	-1.51	(-7.08, 4.07)	0.280	0.596
McMaster-UTC *vs.* MF-UTC	598	0.23	(-4.71, 5.17)	0.008	0.927
McMaster-Day0 *vs.* MF-Day 0	573	0.68	(-5.36, 6.72)	0.049	0.826

Table 6: Comparison of FECRT methods using General Estimating Equations (GEE)

Generalised Estimating Equation (GEE) methods were used to investigate the differences in estimated efficacies using two methods/approaches with population-averaged panel-data. The GEE is a method for analysing repeated-measures regression model that takes into account the correlation of the repeated measures within a farm under minimal assumptions. Results are shown in Table 2. The dependent variable was estimated efficacies and explanatory variables were farms and methods/approaches used to measure the efficacies estimated using faecal egg counts data, these included McMaster-Day0, McMaster-UTC, MF-Day0 and MF-UTC.

Exchangeable method was used as the correlation structure with Gaussian (normal) family. All analyses were performed in R (R Core Team, v3.6.3, 2020) using geepack library (Version 1.31.; Højsgaard et al., 2006; Yan and Fine, 2004; Yan, 2002) using geeglm function. The coefficients (±se) and statistical probabilities for comparisons between four groups (across all drugs and all farms included) are presented in Table 1.

The strongest correlations between methods were when comparison was made between protocols with the same control. McMaster method compared to Mini-FLOTAC, both with untreated controls on Day 14 had a correlation of p=0.927. McMaster method compared to Mini-FLOTAC, both with Day 0 controls had a correlation of p=0.826.

Discussion

This study had two main objectives. The primary one was to compare two different protocols (untreated control group vs Day 0 control group) as well as two different FEC methods (15 individual samples analysed with McMaster at a sensitivity of 50 and 15 samples pooled into three analysed with Mini-FLOTAC at a sensitivity of five). A secondary objective was to obtain recent drench efficacy data against gastrointestinal parasites on Australian sheep farms.

The first part of the project compared the performances between the McMaster and Mini-FLOTAC method. Overall, there was a high level of agreement between results obtained from both methods, 67.8 % samples having FEC from both tests differing by less than 101 epg and concordance correlation coefficient 0.97 (95 % CI 0.96-0.98).

Screening tests are, by their nature, used to detect disease in clinically normal animals with the goal of early detection of disease benefiting the animal (Doohoo, Martin & Stryhn 2003, p. 86). Even though FECs have been found to be well correlated for *H. contortus*, the correlation for the other major species of worms is less precise. However, Mavrot et al. (2015) report that in a meta-analysis that examined 9 slaughter studies of lambs, there was a strong positive correlation (Spearmans's rho = 0.71) between pre-slaughter worm egg counts and total worm count, regardless of worm species.

It is also important to remember that FEC is not necessarily an accurate measure of worm burden, as it may significantly underestimate in cases with hypobiotic worm populations (Sutherland & Scott 2010), or fresh infestations prior to egg production (Besier et al. 2016).

However, when evaluating the efficacy of a drench, test sensitivity is vital. Using the McMaster method, it is not possible to accurately determine FECs in the lower FEC range. Given its higher sensitivity this may be possible using Mini-FLOTAC. Of the 16 samples for which McMaster reported an epg of 0, Mini-FLOTAC reported a epg of 0 in 5, a FEC of <50 in 7, FEC 50-100 in 2 and FEC >100 in 2.

WAAVP guidelines currently recommend that 15 animals per group with an average FEC of 300 EPG be used for FEC reduction tests. This equates to an average of 6 eggs counted per sample using the McMaster technique. There are inherent health risks associated with allowing worm burdens to escalate to that level (Besier et al. 2016), especially considering that 300 epg is the average and due to the anticipated distribution of worm egg counts in a mob of sheep being a negative binomial, there are likely animals in the mob with significantly higher FEC.

Analytic sensitivity (inversely related to the magnitude of the number that the eggs counted must be multiplied by) can be improved by increasing the sample weight or the number of chambers used. Increasing the number of chambers counted in the McMaster technique increases the time and equipment required to run each test (Hutchinson 2009). The other option is to increase the sample weight; however, this is not desirable using the McMaster technique, as the amount of suspended debris increases with sample weight. This latter drawback is circumvented in the Mini-FLOTAC by waiting 10 minutes for the eggs to float to the top, then rotating the top of the device 90° to separate the layer of solution containing the eggs from the layer containing the faecal debris (Ianniello et al. 2015).

The time required to process samples is slightly longer for Mini-FLOTAC than McMaster. This can be attributed to 3 main stages: loading, settling and counting. The mean loading time for the Mini-FLOTAC technique was 3.6 times higher than that of the McMaster technique. For each sample, only one 0.5 mL chamber was loaded using the McMaster technique, whereas the Mini-FLOTAC required the filling of two chambers, each of which held 1 mL; the total volume to be loaded was therefore four times higher for the Mini-FLOTAC.

Unlike the McMaster technique, the Mini-FLOTAC technique requires 10 minutes settling time prior to translating the top of the device 90 °C to separate the eggs from the remainder of the solution. But when preparing several samples in parallel this time can easily be incorporated in the preparation protocol. The total time required to count eggs in a sample was 4.3 times higher for Mini-FLOTAC than McMaster. This is not entirely surprising, considering an average of 11 times more eggs were counted using the Mini-FLOTAC technique than the McMaster technique. Indeed, it took less time to count 10 eggs using the Mini-FLOTAC technique compared to McMaster. Keeping in mind that only three samples needed to be counted for the mini-FLOTAC approach but 15 individual samples for the McMaster approach, the latter took more time to complete.

On the other hand, it was observed that creating three pools containing five samples each, limits the number of total observation (as in counted eggs) and therefore the statistical power. In similar FECRT studies performed by Dawbuts we have since evaluated the performance of using five pools with each containing three samples and the Mini-FLOTAC method with a sensitivity of 10 epg. Even though the sensitivity was reduced from 5epg to 10 epg, the statistical power of this approach was still superior to performing 15 individual samples at a sensitivity of 50 epg.

Therefore, the pooling of 15 samples into 5 pools of three analysed with Mini-FLOTAC at a sensitivity of 10 epg will be assessed by Dawbuts and evaluated against the protocols in this study.

Impact of variation of Day 0 and Day 14 worm egg counts

One of the difficulties sheep producers face in conducting a drench resistance test is finding a suitable mob. Current guidelines require a starting worm egg count of 300 epg. Screening worm egg count tests are required to select suitable mobs of sheep for a drench resistance test. Often sheep are screened but worm egg counts on the day of testing are vastly different from the screening test. The current analysis shows that

when *Haemonchus* levels are low (<30%), the variation is also low, with observed values at D14 only 149 epg different to Day0. However, when *Haemonchus* levels were high (>30%), the difference between D14 and Day 0 also increased, to 730 epg, p=0.0001.

Insufficient worm egg counts at D0 lead to poor estimates of drench efficacy, while high counts can impact on sheep welfare. It is therefore recommended that in areas where *Haemonchus* is >30%, screening tests are repeated weekly to ensure that the 'sweet spot' of 300epg is achieved reliably for the starting (D0) worm egg count. On properties, where low *Haemonchus* was detected, FECs between the Day 0 control and the Day 14 UTC were not significantly different, indicating that running an UTC is particularly problematic when *Haemonchus* is present.

Drench efficacies

As presumed prior to the start of the survey, the data obtained revealed a lack of efficacy for several active ingredients on almost all farms. Worm populations resistant to at least one single-active drench were present on every farm. On about one-third of the evaluated properties, only the newer anthelmintics still remined effective. The fact that reduction of efficacy of at least 3 out of 5 tested drug classes is present on every farm included, highlights the importance of performing a drench test prior to the envisaged treatment of the flock.

Reliability of larval differentiation

The observation in this study that the macrocyclic lactone class products (abamectin and moxidectin) had reliably high efficacy against *Trichostrongylus* raises an important issue regarding larval identification. Previous summary reports have indicated that resistance to both abamectin and moxidectin were detected in drench tests across eastern Australia, with the national average efficacy of abamectin against *Trichostrongylus* at 88% and the average reported efficacy of moxidectin reported at 95% (Playford et al. 2014).

Using standard light microscopic techniques, *Trichostrongylus* and *Teladorsagia* are difficult to distinguish at the third larval stage, even with precise staining. Although identification of these nematodes is possible using molecular techniques, these have not been commercially successful due to lack of practicality and uptake across the country is low, with only one commercial laboratory currently offering the test (Mark Williamson, pers comm). In this study, all larval identification work was conducted in the same laboratory by an experienced veterinary parasitology technician.

The detection of ML-resistant *Trichostrongylus* would most likely not occur, or occur only when it is actually present, if sheep were tested using molecular larval identification or slaughter studies so that adult worms could be reliably identified. However, widespread use of these methods is unjustifiable because they are costly, inconvenient and in the case of slaughter studies, ethically unacceptable for routine use.

The alternative is for a validation program so that operators conducting larval identification in parasitology laboratories can compare methods and cross validate their findings. This would be an important addition to the current ParaBoss WEC QA program.

Comparison of different faecal egg count reduction test methodologies

Analysis of the differences between methods across 1171 different combinations of active ingredient and worm species show that there are no material differences in efficacy estimation. The practical corollary of this finding is that, since no single method showed superior rigour in estimating efficacy the decision on which method to be used can be based on other features such as cost, practicality and availability.

Conclusions and Recommendations

The adoption of techniques such as the FECRT on Australian sheep farms still remains a relatively challenging task for the industry.

The newly established protocol combined with the more sensitive Mini-FLOTAC method proved to be reliable and easy to follow. The calculation of efficacies was performed using a program which not only calculates the mean efficacy but also the confidence intervals. As the name suggest, those intervals are necessary to interpret the results with confidence. For example, the mean value can be 80% but the confidence interval ranges from 10-100.

That means, that in some animals the efficacy might be good, but in the majority of animals it will be lower than 50%. If the mean efficacy is 80% and the confidence intervals range between 75-85% it means that in almost every animal the average efficacy will be achieved. The fact that farmers were not just given an average efficacy but also presented with the confidence intervals, in combination with the respective explanation, may increase the understanding of their importance. The promotion of this new protocol in projects with similar aims is highly recommended.

The trials outlined in the report above indicate that in the Dawbuts laboratory, Mini-FLOTAC has a high degree of concordance (0.97) with the current method (McMaster). This finding is consistent with the results published from other laboratories in Italy, Germany, Belgium, Sweden, Scotland and the UK. On top of this, Mini-FLOTAC has a higher worm egg recovery rate, precision and accuracy compared to other methods (Scare et al. 2017).

The Mini-FLOTAC method does take slightly more time than the McMaster method per sample. However, greater familiarity in the laboratory and pooling of samples will reduce this time and the benefits of using a more sensitive method weigh this up.

It is recommended that AWI support:

1. Adoption of Mini-FLOTAC in veterinary parasitology laboratories where more sensitive methods provide a benefit e.g. in FECRTs.
2. Promotion of drench resistance testing and awareness of drench resistance as a means of improving flock welfare and farm productivity.
3. Voluntary quality assurance schemes and training to enable all users to achieve repeatable and reliable worm egg count testing as well as larval identification.
4. Extension to promote better diagnostics and worm control in Australian sheep flocks.

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List of Abbreviations and/or Glossary

ABA	Abamectin
AWI	Australian Wool Innovation
BZ	Benzimidazoles
CLO	Closantel
DER	Startect ®
EPG	Eggs Per Gram
FEC	Faecal Egg Count
FECRT	Faecal Egg Count Reduction Test
LEV	Levamisole
MLA	Meat & Livestock Australia
MOX	Moxidectin
MPL	Monepantel (Zolvix®)
UTC	Untreated control group
WEC	Worm Egg Count

Appendix 7- Chart showing values of untreated control samples and percent *Haemonchus* cultured in D0 control samples from trial farms across Australia.

Farm No.	postcode	Day0 D0	Control D14	Diff	ABS Diff	%HcD0	Diff sq	av. 149epg Hc<30%	av. 730epg Hc>30%
3	2631	77	360	283.00	283.00	71	80089		283.00
4	2632	350	1512	1162.00	1162.00	75	1350244		1162.00
5	2632	253	385	132.00	132.00	3	17424	132.00	
6	2630	423	297	126.00	126.00	27	15876	126.00	
8	2277	913	357	556.00	556.00	47	309136		556.00
9	2631	993	403	590.00	590.00	47	348100		590.00
10	2631	430	1543	1113.00	1113.00	86	1238769		1113.00
11	3678	1077	1027	50.00	50.00	91	2500		50.00
12	2631	710	1500	790.00	790.00	93	624100		790.00
13	7140	347	113	234.00	234.00	0	54756	234.00	
18	7209	547	143	404.00	404.00	0	163216	404.00	
19	7190	197	180	17.00	17.00	0	289	17.00	
23	7140	250	80	170.00	170.00	0	28900	170.00	
28	5273	237	330	93.00	93.00	75	8649		93.00
29	5262	313	407	94.00	94.00	0	8836	94.00	
30	5262	147	157	10.00	10.00	0	100	10.00	
32	5271	140	113	27.00	27.00	34	729		27.00
34	5280	213	27	186.00	186.00	9	34596	186.00	
36	6318	253	227	26.00	26.00	0	676	26.00	
38	2631	30	163	133.00	133.00	2	17689	133.00	
39	6294	163	118	45.00	45.00	4	2025	45.00	
41	2630	647	414	233.00	233.00	0	54289	233.00	
42	2632	1713	1117	596.00	596.00	94	355216		596.00
44	2633	643	1877	1234.00	1234.00	98	1522756		1234.00
45	2630	2960	1003	1957.00	1957.00	96	3829849		1957.00
46	2633	223	300	77.00	77.00	12	5929	77.00	
48	2631	2290	3097	807.00	807.00	98	651249		807.00
49	2630	2230	3390	1160.00	1160.00	98	1345600		1160.00
50	2630	1673	550	1123.00	1123.00	96	1261129		1123.00
51	2632	7002	4733	2269.00	2269.00	100	5148361		2269.00
52	2365	290	570	280.00	280.00	100	78400		280.00
53	6395	1013	893	120.00	120.00	0	14400	120.00	
54	5272	897	813	84.00	84.00	22	7056	84.00	
56	5267	600	1083	483.00	483.00	68	233289		483.00
57	6350	147	37	110.00	110.00	0	12100	110.00	
59	6258	250	173	77.00	77.00	57	5929		77.00
62	5272	643	747	104.00	104.00	34	10816		104.00
63	5272	623	1360	737.00	737.00	9	543169	737.00	
64	2794	1063	497	566.00	566.00	94	320356		566.00
65	6392	273	467	194.00	194.00	0	37636	194.00	
67	6392	403	387	16.00	16.00	0	256	16.00	
69	6392	200	193	7.00	7.00	0	49	7.00	
72	6324	883	1003	120.00	120.00	7	14400	120.00	
		808	794	14	432	41	459510	149	730
				1.68%	54%			n=21	n=22