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Comparison of molecular and McMaster microscopy techniques to confirm the presence of naturally acquired strongylid nematode infections in sheep.

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Abstract

Patent strongylid nematode infections were identified using McMaster worm egg counts (WEC) and PCR assays (ITS-2 nuclear ribosomal DNA) to screen genomic DNA extracted directly from lamb faecal samples. Lambs from four different farms in southern Western Australia were sampled rectally on two separate occasions, with McMaster WECs and PCRs conducted on a total of 858 samples. Negative controls (n=96) (WEC <50 eggs per gram [epg]) and positive controls (n=96) (faecal samples spiked with a 100µL suspension of third-stage larvae (L3) containing approximately equal proportions of Teladorsagia circumcincta, Trichostrongylus colubriformis, Haemonchus contortus, Oesophagostomum spp. and Chabertia ovina) were generated. All control samples amplified in accordance with positive controls. High levels of agreement (Kappa values ≥ 0.93) were identified between the two diagnostic tests. PCRs detected an additional 2.0% of samples as strongylid-positive but there was no significant difference in the number of strongylid-positive samples identified using PCR or McMaster WEC.

Keywords: sheep; strongylid nematodes; PCR; diagnosis; DNA extraction; McMaster Faecal Worm Egg Count.
Strongylid nematodes are an important cause of income loss in sheep enterprises as a consequence of reduced flock productivity and increased costs associated with anthelmintic treatments. In southern Australia, the most economically important sheep strongylid genera are *Teladorsagia circumcincta*, *Trichostrongylus* spp. and *Haemonchus contortus*, with *Chabertia ovina*, *Oesophagostomum* spp. and *Nematodirus* spp. also commonly present as part of mixed infections [1]. Accurate diagnosis and quantification of strongylid infection is pivotal for both effective control programs and monitoring anthelmintic treatment efficacy [1, 2]. Species-specific diagnosis has important implications for anthelmintic treatment decisions, surveillance of anthelmintic resistance and monitoring strongylid epidemiology demographics in different geographical locations.

The McMaster faecal worm egg count (WEC) flotation technique [3] is widely utilised for the diagnosis and quantification of strongylid worm infections in sheep. The WEC technique is a useful indicator for predicting patent worm burdens, particularly in younger sheep [4], but cannot distinguish between strongylid species without the use of larval culture. Larval cultures are time consuming, require skilled laboratory staff, depend on strictly controlled culture conditions (temperature and humidity) to prevent a species biased culture and require relatively large volumes of faecal material. As a result, larval cultures are typically only performed on pooled faecal samples [5].

Polymerase chain reaction (PCR) assays have been developed to detect patent strongylid species infections in sheep by targeting genetic markers within the internal transcribed spacer (ITS-2) region of nuclear ribosomal DNA [6]. These assays have been used to detect strongylid DNA extracted from worm eggs, following egg purification from sheep faeces by sodium nitrate flotation and column-purification [6, 7] and more recently genomic DNA extracted directly from unprocessed faeces [8]. Comparisons of the level of agreement between the McMaster WEC and PCR diagnostic tests, which screen genomic
DNA extracted directly from faeces, have not been reported. Therefore, the aim of this study was to utilise a commercial DNA extraction kit to extract genomic strongylid DNA from unprocessed sheep faeces and to compare the level of agreement between PCR assays with the McMaster WEC flotation method for identifying patent strongylid infections in lambs.

This experiment was approved by the Murdoch University Animal Ethics Committee (permit R2369/10). Faecal samples were collected from a total of 429 lambs located on four sheep farms in southern Western Australia (Boyup Brook, Kojonup, Pingelly and Arthur River), in a geographical region with a Mediterranean environment [9]. Average annual rainfall for the four sites ranged between 420 – 550mm per annum.

A total of 858 faecal samples were collected over two separate sampling occasions when lambs were approximately 2 – 3 months old (first sampling) and 4 – 5 months old (second sampling). Lambs at Pingelly and Arthur River were treated with 12mg abamectin (Virbamec Oral Plus Selenium, Virbac Australia), while those lambs at Boyup Brook and Kojonup were treated with 12.5mg of moxidectin (Cydectin Weanerguard with Selenium and Vitamin B12, Virbac Australia) immediately after the first sampling. The number of days between the first and second samplings was 35, 29, 40 and 31 for Boyup Brook, Kojonup, Pingelly and Arthur River farms respectively.

Negative control faecal samples were collected rectally from 6-year-old Merino ewes seven days post-treatment with 2.5mg/kg Monepantel (Zolvix, Novartis Australia) administered according to the heaviest ewe live weight.

Faecal worm egg counts were performed within two days of collection using a modified McMaster WEC flotation technique with a detection level/multiplication factor of
50 eggs per gram (epg) [10]. Larval cultures were performed according to the Australian Standard Diagnostic Techniques for Animal Diseases Manual [10] on pooled faecal samples from only the Boyup Brook and Kojonup flocks.

The PCR-positive controls were created using strongylid third-stage larvae (L₃) suspensions. Larvae were collected from larval cultures of fresh sheep faeces and 200ul aliquot L₃ suspensions were collected for each of the following strongylid species; *T. circumcincta*, *Trichostrongylus colubriformis*, *H. contortus*, *C. ovina* and *Oesophagostomum venulosum*. Each larvae suspension from the five strongylid genera had DNA extracted from larval tissues (Fig. 1[A]) [11]. Suspensions of 100µL were created to contain equal proportions of strongylid genera L₃ and used to spike the positive controls (Fig. 1[B]). Unspiked, negative controls (ewe faecal samples providing three consecutive WEC<50epg; n=96) and spiked positive controls (ewe faecal samples providing three consecutive WEC<50epg, spiked with a 100µL suspension containing equal proportions of strongylid species L₃; n=96) were generated to ascertain whether PCR inhibition was observed for any of the strongylid species-specific PCR assays (Fig. 1[B]).

Genomic DNA was extracted from lamb faecal samples within seven days of collection by using Power Soil DNA Isolation Kits (MolBio, West Carlsbad, California, USA) (Fig. 1[C]). A sub-sample comprising of 250–300mg was taken from the centre of each faecal sample. Previously reported minor modifications to the manufacturer’s protocol were made [12].

Conventional PCR assays were performed for each of the following strongylid species; *T. circumcincta*, *Trichostrongylus spp.*, *H. contortus* and *C. ovina*, as described by a previous study [6]. Individual species-specific forward primers (TEL, TRI, HAE and CHO) designed for the second internal transcribed spacer (ITS-2) of ribosomal DNA and the reverse primer (NC2) located at the 5’-region of the 28S rRNA gene, were used to detect
each of the above species. The primer pair TRI-NC2, was capable of detecting all four
major *Trichostrongylus* species (*T. colubriformis*, *T. axei*, *T. vitrinus* and *T. rugatus*) and in
addition *Oesophagostomum columbianum* and *Oesophagostomum venulosum* [6]. The
thermocycling conditions used for each PCR assay are presented in an earlier study [6].
The PCR reactions were performed using 1µL of DNA in a 25µL reaction containing 1 x
PCR buffer, 2.5mM MgCl₂, 0.4mM dNTPs, 0.80µM of each primer and 0.04U/µL of *kapa*
Taq DNA Polymerase (Kapa Biosystems, Cape Town, South Africa) [8]. Negative (no DNA
template added) and positive (genomic DNA from L₃ tissue extractions) controls were
included for all strongylid speices PCR assays.

For any samples that tested McMaster WEC-positive and PCR-negative, a 10µL
genomic DNA extract of the sample was spiked with 1µL aliquots of DNA from each of the
five strongylid species (isolated from the L₃ DNA tissue extractions). From these 15µL
spiked mixtures, a 1µL aliquot was then re-screened with each strongylid species-specific
PCR assay to detect any inhibition.

The PCR 1% agarose gel product for samples that tested PCR-positive and
McMaster WEC-negative (n=17 at the first sampling and n=7 at the second sampling)
were purified using an UltraClean™ DNA Purification Kit (MolBio, West Carlsbad,
California, USA) and sequenced using an ABI Prism™ Terminator Cycle Sequencing Kit
(Applied Biosystems, Foster City, California, USA) on an Applied Biosystem 3730 DNA
Analyzer. Sequence searches were conducted using BLAST
(http://blast.ncbi.nlm.nih.gov/Blast.cgi) and nucleotide sequences were analysed using
Chromas lite version 2.0 (http://www.technelysium.com.au). Subsequently these searches
were aligned with reference genotypes to confirm positive identification for either *H.*
contortus, *T. circumcincta*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *C ovina,*
*Oesophagostomum venulosum* or *Oesophagostomum columbianum* (GenBank accession
numbers AJ57746.1, AJ577463.1, AY439026.1, EF427624, AY439021.1, Y10790.1 and
AJ006150, respectively) using Clustal W (http://www.clustalw.genome.jp).

Statistical analyses were performed using SPSS Statistics 17.0 (Statistical Package
for the Social Sciences) for Windows (SPSS inc. Chicago, USA). The sensitivity and
specificity between PCR and McMaster WEC results were calculated for faecal samples
collected rectally from lambs on-farm, with their infection status unknown. Kappa statistic
was calculated at each sampling to assess the level of agreement between the McMaster
WEC and PCR test results. Either Pearson’s chi-squared test or Fisher’s exact two-sided
test for independence was used to determine if significant differences existed between the
proportions of mixed infections detected at each sampling within each flock. The mean
number of strongylid species detected from each lamb was calculated using arithmetic
means. Differences in the mean number of strongylid species detected per lamb were
performed by univariate analyses (ANOVA) and least significant difference post-hoc tests.

1. PCR assays diagnostic sensitivity – spiked samples

The PCR assays had a diagnostic sensitivity of 100% for the 96 spiked positive
controls and a specificity of 100% (95% CI: 96.2 – 100%) for the negative controls (n=96)
providing three consecutive WEC<50epg.

2. Agreement between PCR assays and McMaster WEC

There was a high level of agreement between PCR and McMaster WEC with Kappa
values of 0.93 (90.4 – 95.4%) at the first sampling and 0.97 (94.6 – 98.2%) at the second
sampling (Fig. 2[A]). For identifying lambs with WEC≥50 epg, the PCR assays had a
sensitivity of 99.7% (98.2 – 100%, n=301) and 100% (98.3 – 100%, n=221) and a
specificity of 91.4% (85.1 – 95.6%, n=128) and 96.6% (93.2 – 98.6, n=208) at the first and second samplings, respectively (Fig. 2[A]).

One sample (0.1%) was McMaster WEC-positive (50 epg) and PCR-negative. Separate spiked DNA extracts from this same sample were screened by PCR for the respective strongylid genera DNA to test for inhibition. This spiked sample amplified in accordance with positive controls for all species-specific PCRs.

Eighteen (2.1%) samples that were McMaster WEC-negative and PCR-positive were re-screened by PCR for all five strongylid species and sequenced. The sequenced products 

3. Strongylid nematode prevalence and species detected by PCR and larval culture

Overall a total of 502/858 (58.5%) samples were McMaster WEC-positive (WEC≥50 epg) and 519/858 (60.5%) samples PCR-positive for at least one strongylid species (Table 1). There was no significant difference in the number of strongylid-positive samples identified by each of the two diagnostic tests. Overall prevalence of patent strongylid infections across all four flocks was 358/429 (83%) detected by PCR (lambs positive for at least one strongylid species on at least one sampling) and 351/429 (81%) detected by McMaster WEC (lamb WEC≥50 epg on at least one sampling).

Across all four flocks, the strongylid species most commonly identified by PCR were 

T. circumcincta (40.2%), Trichostrongylus spp. (24.7%), C. ovina (15.6%) and Oesophagostomum spp. (10.1%), with H. contortus only identified from Boyup Brook lambs (Table 1, Fig. 2[B]). Larval culture results for Boyup Brook and Kojonup flocks are shown in Fig. 2[C]. The total numbers of strongylid species identified by PCR per lamb are
shown in Fig. 2[D]. Mixed infections were identified in 35.3% and 17.0% of lambs at the first and second sampling, respectively. The most common mixed infection for all flocks was that of *Trichostrongylus spp* and *T. circumcincta*.

This study utilised molecular PCR assays that have been previously described [6] for detecting patent strongylid nematode infections from genomic DNA that was extracted directly from unprocessed sheep faeces. Other studies have demonstrated that these PCR assays can be used to identify naturally acquired strongylid infections following separation of strongylid worm eggs from faeces, by using sodium nitrate flotation and column-purification [7]. However in the present study, direct extraction of DNA from faeces by the use of a commercial DNA extraction kit successfully identified strongylid species-specific infection. The PCR diagnostic assays successfully identified all strongylid genera in faecal samples that were spiked with a L3 suspension containing all five species. No PCR inhibition was detected in any of the L3 spiked faecal samples for all strongylid species-specific PCRs. Negative controls never tested positive for any of the strongylid species.

There was a high level of agreement between McMaster WEC and PCR diagnostic test results, suggesting that PCRs detecting DNA extracted directly from lamb faeces had a similar capacity to the traditional McMaster WEC technique for detecting patent strongylid infections. Faecal samples were collected from lambs on commercial sheep farms and post-mortem total worm count examinations were not performed, so the infection status of individual lambs was unknown. Studies that include total worm counts are necessary to confirm the PCR capacity for detecting patent strongylid infections and also to determine sensitivity and specificity for identifying infected sheep. If PCR is shown to have a higher level of sensitivity compared to traditional methods, then there may be an emerging recommendation to incorporate PCR assays for anthelmintic efficacy testing
protocols where a high degree of precision for identifying infected sheep and strongylid species identification is required.

One (0.1%) sample was McMaster WEC-positive and PCR-negative. This may reflect the presence of strongylid species not screened for by PCR, but which have eggs indistinguishable from those of other strongylid species such as *Cooperia spp.*, which has been reported in worm populations in southern Australian sheep flocks [13]. Another possible reason for this discrepancy is the uneven distribution of strongylid worm eggs within the faecal mass sample [14].

Eighteen (2.1%) samples were identified as PCR-positive but McMaster WEC-negative. False PCR-positive identification of patent strongylid infections appears unlikely, with a recent study finding that 100 sheep with a very low risk of nematode infection (housed indoors and treated with anthelmintics) never tested positive in any species-specific PCR assays [7]. Lower detection limits for McMaster WEC (25 epg, 20 epg or 10 epg) could be compared to those PCR assays, to establish whether lower WEC detection limits decrease the number of samples identified as PCR-positive and McMaster WEC-negative.

The PCRs are capable of detecting DNA from strongylid species in sheep faeces, predominantly from worm eggs laid by established, mature females [6]. However, DNA was extracted directly from faeces in this study and the PCR diagnostic assays were therefore presumably incapable of differentiating strongylid DNA originating from patent and non-patent infections (immature larvae and dead worm tissue present in faeces). Some lambs tested negative for strongylid infections while grazing pastures infested with larvae and this suggests that strongylid worm eggs are the likely main source of DNA in faecal DNA extractions. Neither McMaster WEC, nor PCR are capable of distinguishing between viable and non-viable strongylid eggs.
Although PCRs offer some advantages over traditional methods for identifying strongylid infected sheep, the assays are not quantitative and WECs are still required to quantify the magnitude of infections. The fact that PCRs are incapable of differentiating between patent and non-patent origins of DNA extracted directly from faeces, presents a possible disadvantage in detecting patent strongylid infections. However this attribute may be advantageous for the early detection of pathogenic strongylid larvae species infesting those pastures with grazing sheep. Another disadvantage of the PCRs is that each strongylid species-specific PCR assay (except Oesophagostomum spp.) must be conducted separately and as a result greater cost would be incurred associated with use of more PCR reagents. Further modifications of the individual species-specific PCRs into a single, multiplex, quantitative PCR assay would help facilitate the use of PCR for the routine diagnosis of helminthosis in sheep.

Direct DNA extraction allows for other internal pathogens (parasites, bacteria or viruses) to be screened for by utilising a similar molecular approach as conducted in this study. Moreover, the main advantage of PCR assays over McMaster WEC is that they can differentiate strongylid genera present in faeces by using smaller sample volumes and in a shorter time frame than traditional larval cultures. The PCR assays offer the ability to screen individual sheep faecal samples with low worm burdens, making it possible to detect the proportion of a flock which harbour specific strongylid species. In contrast, larval culture differentiations are typically performed on pooled samples to determine the proportion of each species present in an overall strongylid worm population. Larval cultures have also been previously reported to have a biased tendency to identify particular larval species [5]. Rapid and accurate identification of strongylid species infections by PCR may be useful for the expeditious diagnosis of highly pathogenic strongylids, such as H. contortus, which are capable of causing high mortality rates in
susceptible sheep [15]. For a true assessment and evaluation of the PCR assays against larval cultures, larval cultures would be necessary for each individual faecal sample.

Another diagnostic method utilised for strongylid species-specific identification is the lectin binding assay that differentiates worm eggs using genus-specific carbohydrates on the surface of eggs [16]. This method has proved to be useful for the detection of *H. contortus* and *Trichostrongylus spp.* infections in sheep, with strong correlations found when compared to those observed in larval culture [16]. At present, lectins specific for *T. circumcincta, Oesophagostomum spp.* or *Chabertia ovina*, have not been identified. The time taken to conduct lectin binding assays utilising sugar centrifugation methods (which reduces egg purification time from faeces) [17] is similar to that of PCR. However, PCR can achieve higher sample throughputs by using 96-well PCR plates and also provide genomic DNA available for the testing of a wider range of pathogens [6, 12]. More recently, a faecal occult blood assay utilising a commercial “*Haemonchus* Dipstick Test” has been developed. Although having a short processing time (~30 minutes) and capacity to be processed on-farm (rather than in a laboratory), the test is not quantitative and both false positive and negative results have been reported [18].

In conclusion, the objective of this study was to compare the level of agreement between McMaster WEC and PCR assays (screening genomic DNA extracted directly from faeces utilising a commercial DNA extraction kit) in identifying patent strongylid infections in lambs. No PCR inhibition was detected in spiked faecal samples and unspiked negative control faecal samples never tested PCR-positive. There was a high level (≥0.93) of agreement between PCR and McMaster WEC test results for identifying strongylid positive faecal samples. Validation of PCR and WEC results against post-mortem total worm count results, along with further modifications of the individual species-
specific PCRs into a single, multiplex, quantitative PCR assay, are both required to facilitate the use of PCR for routine diagnosis of helminthosis in sheep.

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References


Comparison of McMaster WEC (microscopy) and PCR diagnostic assays for the detection of patent strongylid infections in four lambs flocks.

<table>
<thead>
<tr>
<th>Site location</th>
<th>Total samples tested (n)</th>
<th>Mean ± SEM (epg)</th>
<th>Range (epg)</th>
<th>positive (n)*</th>
<th>Total</th>
<th>H. contortus</th>
<th>T. circumcincta</th>
<th>Trichostrongylus spp.</th>
<th>C. ovis</th>
<th>Oesophagostomum spp.</th>
<th>Mixed infections a</th>
<th>Mean number of species detected per lamb ± SE</th>
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<tr>
<td>Boyup Brook</td>
<td>First sampling 128</td>
<td>100 ± 13</td>
<td>0 – 750</td>
<td>81</td>
<td>84</td>
<td>9</td>
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<td>14</td>
<td>13</td>
<td>44 (34.4%)</td>
<td>1.11 ± 0.09*</td>
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<td></td>
<td>Second sampling 128</td>
<td>77 ± 12</td>
<td>0 – 700</td>
<td>59</td>
<td>62</td>
<td>8</td>
<td>50</td>
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<td>10</td>
<td>4</td>
<td>36 (28.1%)</td>
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<td>Kojonup</td>
<td>First sampling 72</td>
<td>28 ± 5</td>
<td>0 – 150</td>
<td>26</td>
<td>27</td>
<td>0</td>
<td>23</td>
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<td>8</td>
<td>4</td>
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<td>Second sampling 72</td>
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<td>0 – 150</td>
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<td>0</td>
<td>18</td>
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<td>10 (13.9%)</td>
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<td>73 ± 13</td>
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<td>42</td>
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<td>Second sampling 121</td>
<td>48 ± 7</td>
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<td>65</td>
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<td>48</td>
<td>16</td>
<td>8</td>
<td>6</td>
<td>12 (9.9%)</td>
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<td>Total (%)</td>
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<td>-</td>
<td>-</td>
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<td>519 (60.5%)</td>
<td>17 (2.0%)</td>
<td>345 (40.2%)</td>
<td>212 (24.7%)</td>
<td>134 (15.6%)</td>
<td>87 (10.1%)</td>
<td>223 (26.0%)</td>
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*Values in rows with different superscripts are significantly different within each farm (P<0.05).

**McMaster WEC-positive samples had a WEC≥50 eggs per gram (epg).

*a = mixed infections: lambs positive for two or more of the strongylid species detected by PCR diagnostic assays.
Figure 1. Methodology utilised to screen PCR positive controls [A], spiked and unspiked ewe control faecal samples [B] and on-farm lamb test samples [C].
Figure 2. [A] Comparison of the McMaster WEC (microscopy) and PCR diagnostic assay for the identification of strongylid positive or negative faecal samples. [B] Number of lambs PCR-positive for each of the five strongylid species. [C] Larval culture results from pooled faecal samples. [D] Number of strongylid species identified per lamb by PCR.