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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 (L_3) 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.

30 Strongylid nematodes are an important cause of income loss in sheep enterprises
31 as a consequence of reduced flock productivity and increased costs associated with
32 anthelmintic treatments. In southern Australia, the most economically important sheep
33 strongylid genera are *Teladorsagia circumcincta, Trichostrongylus spp.* and *Haemonchus*
34 *contortus,,* with *Chabertia ovina, Oesophagostomum spp.* and *Nematodirus spp.* also
35 commonly present as part of mixed infections [1]. Accurate diagnosis and quantification of
36 strongylid infection is pivotal for both effective control programs and monitoring
37 anthelmintic treatment efficacy [1, 2]. Species-specific diagnosis has important implications
38 for anthelmintic treatment decisions, surveillance of anthelmintic resistance and monitoring
39 strongylid epidemiology demographics in different geographical locations.

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

48 Polymerase chain reaction (PCR) assays have been developed to detect patent
49 strongylid species infections in sheep by targeting genetic markers within the internal
50 transcribed spacer (ITS-2) region of nuclear ribosomal DNA [6]. These assays have been
51 used to detect strongylid DNA extracted from worm eggs, following egg purification from
52 sheep faeces by sodium nitrate flotation and column-purification [6, 7] and more recently
53 genomic DNA extracted directly from unprocessed faeces [8]. Comparisons of the level of
54 agreement between the McMaster WEC and PCR diagnostic tests, which screen genomic

55 DNA extracted directly from faeces, have not been reported. Therefore, the aim of this
56 study was to utilise a commercial DNA extraction kit to extract genomic strongylid DNA
57 from unprocessed sheep faeces and to compare the level of agreement between PCR
58 assays with the McMaster WEC flotation method for identifying patent strongylid infections
59 in lambs.

60

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

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

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

77 Faecal worm egg counts were performed within two days of collection using a
78 modified McMaster WEC flotation technique with a detection level/multiplication factor of

79 50 eggs per gram (epg) [10]. Larval cultures were performed according to the Australian
80 Standard Diagnostic Techniques for Animal Diseases Manual [10] on pooled faecal
81 samples from only the Boyup Brook and Kojonup flocks.

82 The PCR-positive controls were created using strongylid third-stage larvae (L_3)
83 suspensions. Larvae were collected from larval cultures of fresh sheep faeces and 200ul
84 aliquot L_3 suspensions were collected for each of the following strongylid species; *T.*
85 *circumcincta, Trichostrongylus colubriformis, H. contortus, C. ovina* and
86 *Oesophagostomum venulosum*. Each larvae suspension from the five strongylid genera
87 had DNA extracted from larval tissues (Fig. 1[A]) [11]. Suspensions of 100µL were created
88 to contain equal proportions of strongylid genera L_3 and used to spike the positive controls
89 (Fig. 1[B]). Unspiked, negative controls (ewe faecal samples providing three consecutive
90 WEC<50epg; n=96) and spiked positive controls (ewe faecal samples providing three
91 consecutive WEC<50epg, spiked with a 100µL suspension containing equal proportions of
92 strongylid species L_3; n=96) were generated to ascertain whether PCR inhibition was
93 observed for any of the strongylid species-specific PCR assays (Fig. 1[B]).

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

99 Conventional PCR assays were performed for each of the following strongylid
100 species; *T. circumcincta, Trichostrongylus spp., H. contortus* and *C. ovina*, as described by
101 a previous study [6]. Individual species-specific forward primers (TEL, TRI, HAE and CHO)
102 designed for the second internal transcribed spacer (ITS-2) of ribosomal DNA and the
103 reverse primer (NC2) located at the 5'-region of the 28S rRNA gene, were used to detect

104 each of the above species. The primer pair TRI-NC2, was capable of detecting all four
105 major *Trichostrongylus* species (*T. colubriformis, T. axei, T. vitrinus* and *T. rugatus*) and in
106 addition *Oesophagostomum columbianum* and *Oesophagostomum venulosum* [6]. The
107 thermocycling conditions used for each PCR assay are presented in an earlier study [6].
108 The PCR reactions were performed using 1µL of DNA in a 25µL reaction containing 1 x
109 PCR buffer, 2.5mM $MgCl_2$, 0.4mM dNTPs, 0.80µM of each primer and 0.04U/µL of *kapa*
110 Taq DNA Polymerase (Kapa Biosystems, Cape Town, South Africa) [8]. Negative (no DNA
111 template added) and positive (genomic DNA from L_3 tissue extractions) controls were
112 included for all strongylid speices PCR assays.

113 For any samples that tested McMaster WEC-positive and PCR-negative, a 10µL
114 genomic DNA extract of the sample was spiked with 1µL aliquots of DNA from each of the
115 five strongylid species (isolated from the L_3 DNA tissue extractions). From these 15µL
116 spiked mixtures, a 1µL aliquot was then re-screened with each strongylid species-specific
117 PCR assay to detect any inhibition.

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

129 numbers AJ57746.1, AJ577463.1, AY439026.1, EF427624, AY439021.1, Y10790.1 and
130 AJ006150, respectively) using Clustal W (http://www.clustalw.genome.jp).

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

142

143 **1. PCR assays diagnostic sensitivity – spiked samples**

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

147 **2. Agreement between PCR assays and McMaster WEC**

148 There was a high level of agreement between PCR and McMaster WEC with Kappa
149 values of 0.93 (90.4 – 95.4%) at the first sampling and 0.97 (94.6 – 98.2%) at the second
150 sampling (Fig. 2[A]). For identifying lambs with WEC≥50 epg, the PCR assays had a
151 sensitivity of 99.7% (98.2 – 100%, n=301) and 100% (98.3 – 100%, n=221) and a

152 specificity of 91.4% (85.1 – 95.6%, n=128) and 96.6% (93.2 – 98.6, n=208) at the first and
153 second samplings, respectively (Fig. 2[A]).

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

158 Eighteen (2.1%) samples that were McMaster WEC-negative and PCR-positive
159 were re-screened by PCR for all five strongylid species and sequenced. The sequenced
160 products *T. circumcincta* (n=3), *Trichostrongylus colubriformis* (n=2), *C. ovina* (n=4) and
161 *Oesophagostomum venulosum* (n=8) were aligned with reference sequences on GenBank
162 and were 100% identical.

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

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

171 Across all four flocks, the strongylid species most commonly identified by PCR were
172 *T. circumcincta* (40.2%), *Trichostrongylus spp.* (24.7%), *C. ovina* (15.6%) and
173 *Oesophagostomum spp.* (10.1%), with *H. contortus* only identified from Boyup Brook
174 lambs (Table 1, Fig. 2[B]). Larval culture results for Boyup Brook and Kojonup flocks are
175 shown in Fig. 2[C]. The total numbers of strongylid species identified by PCR per lamb are

176 shown in Fig. 2[D]. Mixed infections were identified in 35.3% and 17.0% of lambs at the
177 first and second sampling, respectively. The most common mixed infection for all flocks
178 was that of *Trichostrongylus spp.* and *T. circumcincta*.

179

180 This study utilised molecular PCR assays that have been previously described [6]
181 for detecting patent strongylid nematode infections from genomic DNA that was extracted
182 directly from unprocessed sheep faeces. Other studies have demonstrated that these PCR
183 assays can be used to identify naturally acquired strongylid infections following separation
184 of strongylid worm eggs from faeces, by using sodium nitrate flotation and column-
185 purification [7]. However in the present study, direct extraction of DNA from faeces by the
186 use of a commercial DNA extraction kit successfully identified strongylid species-specific
187 infection. The PCR diagnostic assays successfully identified all strongylid genera in faecal
188 samples that were spiked with a L_3 suspension containing all five species. No PCR
189 inhibition was detected in any of the L_3 spiked faecal samples for all strongylid species-
190 specific PCRs. Negative controls never tested positive for any of the strongylid species.

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

201 protocols where a high degree of precision for identifying infected sheep and strongylid
202 species identification is required.

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

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

217 The PCRs are capable of detecting DNA from strongylid species in sheep faeces,
218 predominantly from worm eggs laid by established, mature females [6]. However, DNA
219 was extracted directly from faeces in this study and the PCR diagnostic assays were
220 therefore presumably incapable of differentiating strongylid DNA originating from patent
221 and non-patent infections (immature larvae and dead worm tissue present in faeces).
222 Some lambs tested negative for strongylid infections while grazing pastures infested with
223 larvae and this suggests that strongylid worm eggs are the likely main source of DNA in
224 faecal DNA extractions. Neither McMaster WEC, nor PCR are capable of distinguishing
225 between viable and non-viable strongylid eggs.

226 Although PCRs offer some advantages over traditional methods for identifying
227 strongylid infected sheep, the assays are not quantitative and WECs are still required to
228 quantify the magnitude of infections. The fact that PCRs are incapable of differentiating
229 between patent and non-patent origins of DNA extracted directly from faeces, presents a
230 possible disadvantage in detecting patent strongylid infections. However this attribute may
231 be advantageous for the early detection of pathogenic strongylid larvae species infesting
232 those pastures with grazing sheep. Another disadvantage of the PCRs is that each
233 strongylid species-specific PCR assay (except *Oesophagostomum spp.*) must be
234 conducted separately and as a result greater cost would be incurred associated with use
235 of more PCR reagents. Further modifications of the individual species-specific PCRs into a
236 single, multiplex, quantitative PCR assay would help facilitate the use of PCR for the
237 routine diagnosis of helminthosis in sheep.

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

251 susceptible sheep [15]. For a true assessment and evaluation of the PCR assays against
252 larval cultures, larval cultures would be necessary for each individual faecal sample.

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

267 In conclusion, the objective of this study was to compare the level of agreement
268 between McMaster WEC and PCR assays (screening genomic DNA extracted directly
269 from faeces utilising a commercial DNA extraction kit) in identifying patent strongylid
270 infections in lambs. No PCR inhibition was detected in spiked faecal samples and
271 unspiked negative control faecal samples never tested PCR-positive. There was a high
272 level (≥0.93) of agreement between PCR and McMaster WEC test results for identifying
273 strongylid positive faecal samples. Validation of PCR and WEC results against post-
274 mortem total worm count results, along with further modifications of the individual species-

275 specific PCRs into a single, multiplex, quantitative PCR assay, are both required to
276 facilitate the use of PCR for routine diagnosis of helminthosis in sheep.

277

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285

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336

337 **Table 1**

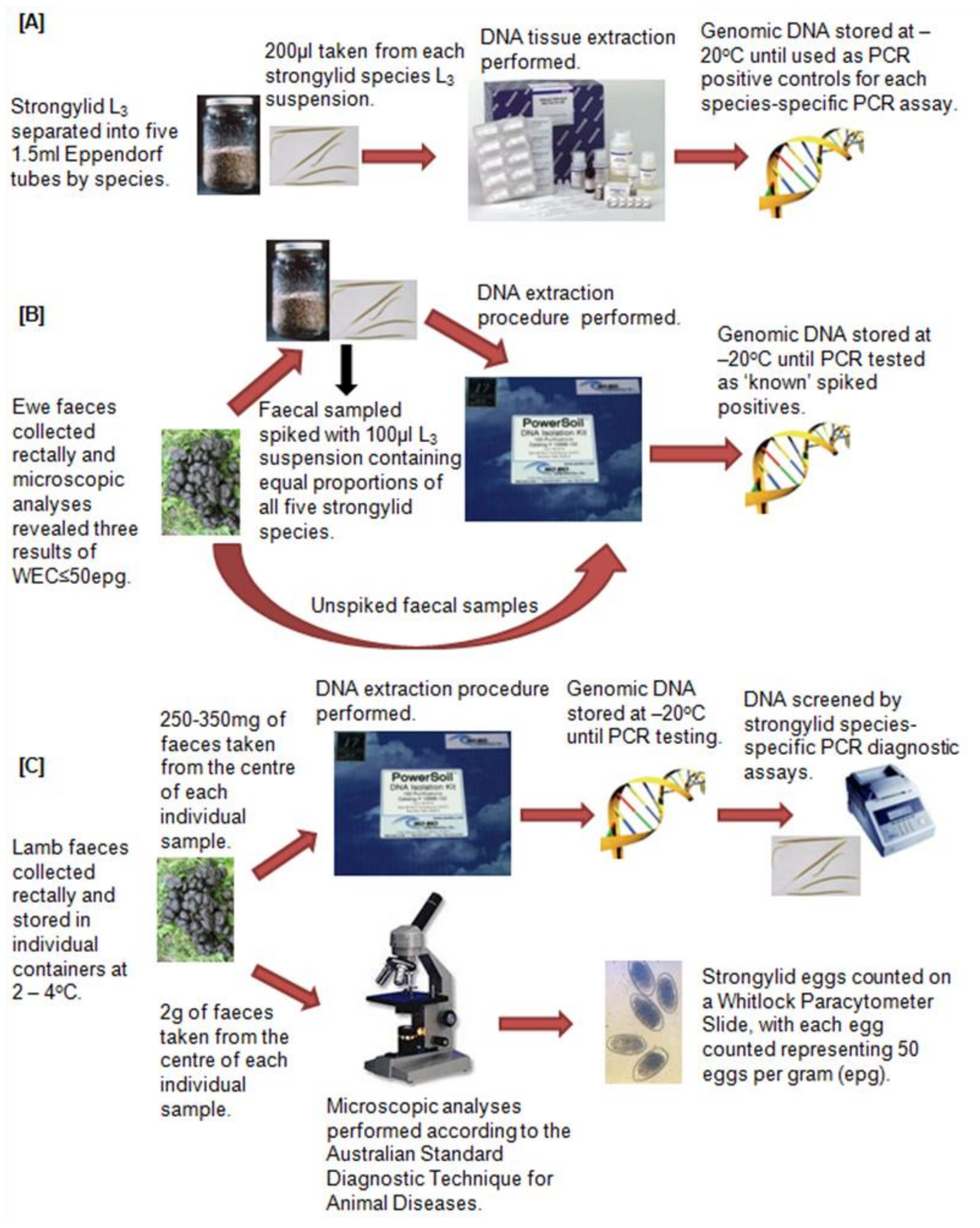
338 Comparison of McMaster WEC (microscopy) and PCR diagnostic assays for the detection of patent strongylid infections in four lambs
339 flocks.

Site location	Total samples tested (n)	McMaster WEC Mean ± SEM (epg)	Range (epg)	**positive (n)***	PCR-positive (n) Total	*H. contortus*	*T. circumcincta*	*Trichostrongylus spp.*	*C. ovina*	*Oesophagostomum spp.*	Mixed infections [a]	Mean number of species detected per lamb ± SE
Boyup Brook												
First sampling	128	100 ± 13	0 – 750	81	84	9	63	44	14	13	44 (34.4%) [A]	1.11 ± 0.09[A]
Second sampling	128	77 ± 12	0 – 700	59	62	8	50	34	10	4	36 (28.1%) [A]	0.83 ± 0.09[B]
Kojonup												
First sampling	72	28 ± 5	0 – 150	26	27	0	23	8	8	4	11 (15.3%) [A]	0.60 ± 0.09[A]
Second sampling	72	21 ± 4	0 – 150	24	24	0	18	11	3	3	10 (13.9%) [A]	0.50 ± 0.09[A]
Pingelly												
First sampling	108	446 ± 45	0 – 3950	102	102	0	73	57	40	22	75 (69.4%) [A]	1.78 ± 0.08[A]
Second sampling	108	73 ± 13	0 – 1100	56	57	0	28	24	15	8	15 (13.9%) [B]	0.70 ± 0.08[A]
Arthur River												
First sampling	121	87 ± 8	0 – 450	92	98	0	42	18	36	27	21 (17.4%) [A]	1.05 ± 0.06[A]
Second sampling	121	48 ± 7	0 – 450	62	65	0	48	16	8	6	12 (9.9%) [A]	0.65 ± 0.09[B]
Total (%)	858	-	-	502	519 (60.5%)	17 (2.0%)	345 (40.2%)	212 (24.7%)	134 (15.6%)	87 (10.1%)	223 (26.0%)	-

340 [AB] Values in rows with different superscripts are significantly different within each farm (P<0.05).

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

342 [a]= mixed infections: lambs positive for two or more of the strongylid species detected by PCR diagnostic assays.



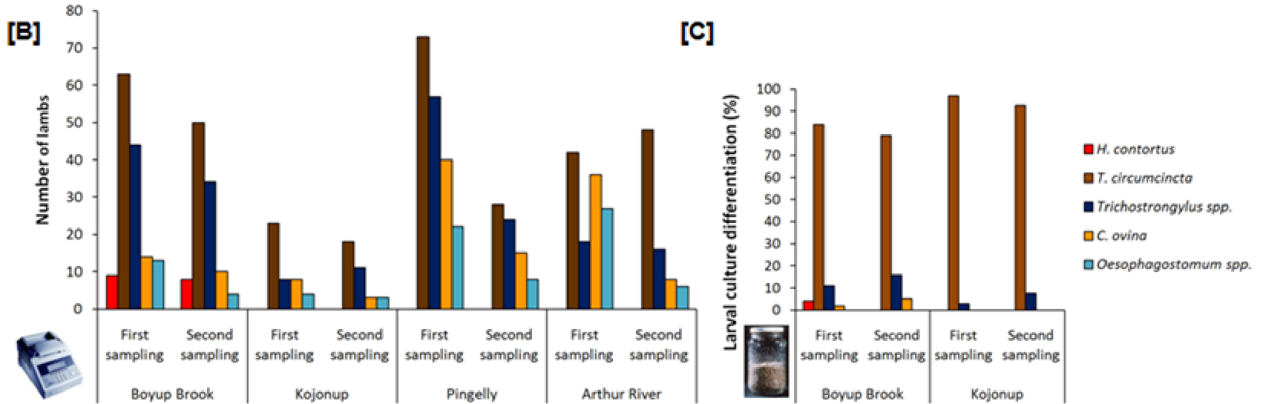
343

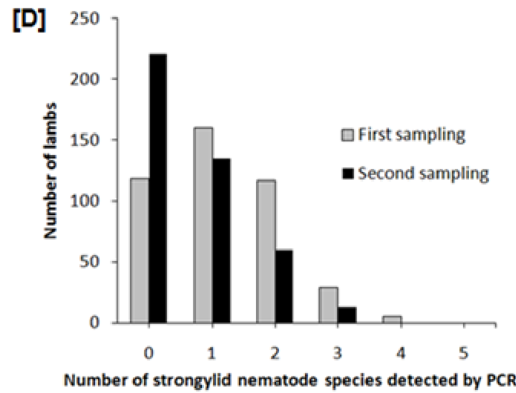
344

345 **Figure 1.** Methodology utilised to screen PCR positive controls [A], spiked and unspiked
346 ewe control faecal samples [B] and on-farm lamb test samples [C].

[A]

	First sampling			Second sampling		
	McMaster WEC			McMaster WEC		
PCR assays	Positive	Negative	Total	Positive	Negative	Total
Positive	300	11	311	201	7	208
Negative	1	117	118	0	221	221
Total	301	128	429	201	228	429





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349 **Figure 2.** [A] Comparison of the McMaster WEC (microscopy) and PCR diagnostic assay
350 for the identification of strongylid positive or negative faecal samples. [B] Number of lambs
351 PCR-positive for each of the five strongylid species. [C] Larval culture results from pooled
352 faecal samples. [D] Number of strongylid species identified per lamb by PCR.