
http://researchrepository.murdoch.edu.au/8677/

Copyright: © 2012 Elsevier B.V..

It is posted here for your personal use. No further distribution is permitted.
Accepted Manuscript

Title: Molecular identification of naturally acquired strongylid infections in lambs—an investigation into how lamb age influences diagnostic sensitivity

Authors: Joshua P.A. Sweeny, Una M. Ryan, Ian D. Robertson, Caroline Jacobson

PII: S0304-4017(12)00010-6
DOI: doi:10.1016/j.vetpar.2012.01.007
Reference: VETPAR 6220

To appear in: Veterinary Parasitology

Received date: 18-10-2011
Revised date: 31-12-2011
Accepted date: 3-1-2012

Please cite this article as: Sweeny, J.P.A., Ryan, U.M., Robertson, I.D., Jacobson, C., Molecular identification of naturally acquired strongylid infections in lambs—an investigation into how lamb age influences diagnostic sensitivity, Veterinary Parasitology (2010), doi:10.1016/j.vetpar.2012.01.007

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Molecular identification of naturally acquired strongylid infections in lambs – an investigation into how lamb age influences diagnostic sensitivity

Joshua P.A. Sweeny *, Una M. Ryan, Ian D. Robertson, Caroline Jacobson

School of Veterinary and Biomedical Sciences, Murdoch University, Western Australia, 6150, Australia

* Corresponding author. Tel.: +61 8 93602495.
E-mail address: J.Sweeny@murdoch.edu.au (J.P.A. Sweeny).
Abstract

Faecal samples ($n = 1155$) were collected from $n = 111$ (Farm A) and $n = 124$ (Farm B) 2-6 week old female lambs on two farms in southern Western Australia across five sampling occasions (spanning 8 months). Genomic DNA was extracted directly from faecal samples and screened by PCR for ITS-2 nuclear ribosomal DNA to detect patent strongylid infections, specifically *Teladorsagia circumcincta*, *Trichostrongylus* spp., *Haemonchus contortus*, *Oesophagostomum* spp. and *Chabertia ovina*. The minimum amount of extracted genomic DNA necessary for successful PCR amplification was 2.0-5.0 pg.

During the five sampling occasions for the two farms, the sensitivities for WEC and PCR identification of strongylid infections varied, with levels of agreement between the two sets of diagnostic results ranging from 85-100%. The strongylid species prevalences were high (90.3-97.3%), with *T. circumcincta* and *Trichostrongylus* spp. the most prevalent species and together they were the most common mixed strongylid infection; *H. contortus* was not identified in either flock. *Teladorsagia circumcincta* was the only species associated with an increased risk of non-pelleted faeces on Farm B, where *T. circumcincta*-positive lambs were 2.3 and 2.6 times more likely to have non-pelleted faeces than negative lambs at the second and final samplings, respectively. The highest strongylid prevalence, mixed strongylid prevalence and mean number of strongylid species detected per lamb coincided with the highest average flock faecal worm egg counts (WECs) on both farms. There was a positive correlation between the number of strongyle species detected per lamb and both WEC and adjusted WEC ($P < 0.01; r^2 0.026-0.591$).

These results indicate that strongylid eggs were likely to be the main source of strongylid DNA in the faecal DNA extracts. Despite the progress made by the molecular approach utilised in this study, it is incapable of distinguishing between patent and non-patent sources of strongylid DNA. However there is potential for further investigation into
the development of a similar molecular procedure which could be used for early larvae
detection on pastures.

*Keywords:* Strongylid nematodes; Sheep; PCR; Faecal DNA extraction; Diagnostic

sensitivity; Faecal worm egg counts
1. Introduction

The management and control of strongylid nematodes in commercial sheep enterprises is critical because of the income loss associated with reduced flock productivity (Sackett et al., 2006). The most economically important sheep strongylid genera in southern Australia are Trichostrongylus spp., Teladorsagia circumcincta and Haemonchus contortus. Chabertia ovina, Oesophagostomum spp. and Nematodirus spp. generally contribute to a lesser extent (Besier and Love, 2003). Accurate and reliable diagnosis of strongylid infections is critical for implementing effective control programmes and for monitoring treatment efficacy (Coles et al., 2006; Woodgate and Besier, 2010). Species-specific diagnosis has important implications for rapid identification of highly pathogenic strongylids, approaches to anthelminthic treatment, geographical surveillance of anthelminthic resistance and for understanding strongylid epidemiology in sheep flocks from different geographical regions.

McMaster faecal worm egg counts (WECs) are a useful guide for detection of patent worm burdens in young sheep (Kingsbury, 1965; McKenna, 1981). However this method cannot distinguish between different strongylid species contributing to an individual WEC without the use of larval culture differentiation. Larval cultures require an experienced microscopist, are time consuming (1 week), have tendencies to produce biased results and demand relatively large volumes of faecal material and so are typically performed on pooled faecal samples only (Dobson et al., 1992).

There has been recent developments regarding species-specific PCR assays, which are capable of detecting different strongylid worm species in DNA extracted from column-purified worm eggs (Bott et al., 2009). However, the use of such species-specific PCRs to screen genomic DNA extracted directly from faeces has not been well examined, with uneven strongylid worm egg distribution in faecal masses (Sinniah, 1982; Tarazona, 1986; Morgan et al., 2005) a concern for an accurate diagnosis. In addition other
challenges with this technique include the small subsample volume of faeces utilised in DNA extractions, the presence of faecal inhibitors (e.g. humic acids and polysaccharides) in samples collected from sheep of different ages and the inability to accurate quantify diagnostic results (Hunt, 2011). Despite these limitations, PCR has advantages over traditional larval culture analysis, including a more rapid and reliable diagnostic identification, an unbiased species identification and the ability to detect different strongylid species. PCR also provides rapid diagnosis of highly pathogenic strongylid species (such as *H. contortus*) and identification of strongylid species, both pre- and post-anthelmintic treatment for efficacy and resistance studies.

A recent study found an overall high level of agreement between PCR and WEC results for the detection of naturally acquired strongylid infections (93%), with DNA extracted directly from faeces (Sweeny *et al.*, 2011). However, this study had the following limitations; it was conducted on only those faecal samples collected from lambs on two separate sampling occasions (separated by ~60 days) and all faecal samplings were collected from lambs on-farm. In addition no investigation was conducted into possible associations/correlations between molecular strongylid species (parasite prevalences) and microscopic WEC results. It has been reported that lambs 1-2 months old, have ~80% of their ingested strongylid third stage larvae (L₃) establish infections, with this percentage declining precipitously as lambs age and acquire immunity to the parasites (Dobson *et al.*, 1990a; Dobson *et al.*, 1990c, b, d). Hence a greater number of sampling occasions would be beneficial to assess whether non-patent sources of DNA (larval tissues passing through the gastrointestinal tract of sheep and expelled in faeces) potentially contribute towards false PCR positive identification. Furthermore, DNA inhibitors are potentially present in lamb faeces, particularly in lairage (livestock holding yards at abattoirs) where time held off feed can increase the concentration of inhibitors in faeces.
The aims of this longitudinal study were to: (1) conduct DNA extractions (using a commercial DNA isolation kit) on individual faecal samples collected from lambs managed under extensive grazing conditions, (2) determine the minimum amount of genomic DNA extracted directly from faeces, for which specific amplification of the ITS-2 region could be achieved, (3) assess the sensitivity of species specific PCRs screening faecal DNA extracts for strongylid worms across multiple sampling occasions (on-farm and lairage), to determine if the levels of agreement between McMaster WEC and PCR are influenced by time of season, lamb age (acquired immunity) and worm burdens, (4) examine whether WEC is associated/correlated with molecular strongylid species results (species prevalences, mixed infections and number of species detected per lamb) and (5) compare the prevalence and distribution of different strongylid genera (H. contortus, T. circumcincta, Trichostrongylus spp., Oesophagostomum spp. and C. ovina) on- and off-farm and whether molecular detection of specific strongylid species is associated with an increased risk of non-pelleted, loose faeces.

2. Materials and methods

2.1 Study sites, animals and experimental protocol

The experiment was approved and supervised by the Murdoch University Animal Ethics Committee (Permit number R2236/09). The two sheep farms in this study were located in southern Western Australia 200-250 km south east of Perth at Pingelly (Farm A: 1500 Ha; 1350 sheep; 32.55° S, 116.87° E) and Arthur River (Farm B: 1250 Ha; 1750 sheep; 33.28° S, 117.01° E) ~150 km apart. These farms experience a Mediterranean climate, with hot, dry summers, cool, wet winters and a predominantly winter rainfall pattern, with an average annual rainfall of 450-500 mm (Hill et al., 2004; Moeller et al., 2008). Winter stocking rates, based on dry sheep equivalents (DSE)/Ha (McLaren, 1997),
were 12 DSE/Ha on Farm A and 10 DSE/Ha on Farm B. An overview of all sampling throughout the study is shown in Table 1.

On each farm, Merino x Suffolk meat lambs were born and raised on one annual pasture paddock consisting primarily of annual ryegrass (*Lolium* spp.) and subterranean clover (*Trifolium subterraneum*). Water was supplied *ad libitum* via a creek (Farm A), dam (Farm B) or by troughs filled from either of these water sources. Supplementary feed, ~100 g/head/day (35% lupins and 65% oats) was provided for each lamb flock after weaning.

At 2-6 weeks of age (day 0 of study), 111 and 124 female lambs from Farms A and B, respectively, were randomly selected and identified utilising unique numbers and radiofrequency ear tags. Faeces were collected directly from the rectum of each lamb at five separate samplings from day 0 (2-6 weeks of age) to 7-8 months of age (Table 1). A total of 107 and 119 lambs from Farms A and B, respectively, were sampled at all five samplings and overall strongylid prevalences were determined from these lambs. Each flock was mustered from their paddock into nearby yards for faecal sampling, except for the final sampling, which took place in lairage facilities following transportation by road to an abattoir for slaughter. Faecal consistency score (FCS) was recorded on a scale of 1-5 (Greeff and Karlsson, 1997).

Faecal samples were collected from each lamb using fresh latex gloves to prevent cross contamination between samples. All faecal samples were placed in individually labelled, airtight 70 mL containers and transported to the laboratory within 6 h of collection. Faecal samples were stored at 2-4 °C and genomic DNA was extracted within 7 days of collection (Yang *et al.*, 2009; Robertson *et al.*, 2010). Lambs on Farm A were consigned for slaughter in two separate groups, the first on day 199 and the second on day 240 of the study. Lambs from Farm B were consigned for slaughter as a single group on day 188 of the study.

### 2.2 Anthelmintic treatment
Each lamb was treated with 12 mg abamectin and 6 mg selenium (Virbamec Oral Plus Selenium, Virbac) before weaning on days 39 (Farm A) and 73 (Farm B).

2.3 Genomic DNA extraction

PCR positive controls for all five strongylid species were created (Table 2). Genomic DNA was extracted from 250-300 mg of each of the 1155 faecal samples that were collected from the identified lambs, using the modified Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc.; 2746 Loker Avenue West; Carlsbad, CA 92010) protocol and stored at -20 °C. In brief, samples were subjected to five freeze-thaw cycles, by which each sample was frozen with liquid nitrogen for 4 minutes and then thawed at 90 °C for 4 minutes. The final elution volume of C6 solution (MO BIO Laboratories) was adjusted to 50 µL to increase the final DNA concentration (Sweeny et al., 2011). DNA extraction positive and negative controls, along with serial dilutions of genomic DNA were generated (Table 2).

2.4 Faecal worm egg counts

Faecal worm egg counts were performed on 2 g faeces from each sample within 2 days of collection using a modified McMaster technique (Lyndal-Murphy, 1993); each egg counted represented 50 epg. At the time of first sampling (day 0), insufficient quantities of faeces were collected from all lambs to perform both DNA extraction and WEC; therefore WEC data were missing for some lambs at this sampling.

2.5 PCR amplification

For each DNA extract, single-step, conventional PCRs were performed for each strongylid nematode (T. circumcincta, Trichostrongylus spp., H. contortus and C. ovina), as described by Bott et al., (2009). Individual species-specific forward primers (TEL, TRI, HAE and CHO) designed for the second internal transcribed spacer (ITS-2) ribosomal DNA and the reverse primer (NC2) located at the 5’ region of the 28S rRNA gene, were used to detect each strongylid species. The primer pair TRI-NC2, was capable of detecting
all four major *Trichostrongylus* spp., including *T. colubriformis*, *T. axei*, *T. vitrinus* and *T. rugatus*, as well as *Oesophagostomum columbianum* and *O. venulosum* (Bott et al., 2009).

For each PCR, a 25 µL reaction mixture contained 1 µL DNA, 1x PCR buffer, 2.5 mM MgCl₂, 0.4 mM each deoxynucleotide triphosphate (dNTP), 0.8 µM each oligonucleotide primer and 0.04 U/µL *Taq* DNA Polymerase (Kapa Biosystems). The PCR thermocycling conditions were as described by Bott et al., (2009).

For any samples that were McMaster WEC positive (≥50 epg) and PCR negative, five separate aliquots (10 µL) of the sample were spiked with 1 µL purified DNA from each of the five strongylid species (T. circumcincta, Trichostrongylus spp., C. ovina, Oesophagostomum spp. and *H. contortus*). A 1 µL aliquot from each of the spiked 15 µL mixtures was then re-screened by each strongylid species-specific PCR assay to test for inhibition. A randomly selected 10 PCR negative samples from each farm, at all five sampling occasions, were re-screened by all five species-specific PCRs, with 0.5µL of DNA template added with 0.5µL of positive control DNA (containing five equal proportions of all five species) and re-screened to test for inhibition.

2.6 Sequence analysis

To confirm accurate identification of *L*₃ from larval cultures, positive control PCR products were sequenced. PCR products from spiked samples and field samples that were PCR positive and McMaster WEC negative (*n* = 5) were purified using the UltraClean DNA Purification Kit (MolBio) and sequenced using an ABI Prism Terminator Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystem 3730 DNA Analyzer. Sequence searches were conducted using BLAST¹ and nucleotide sequences were analysed using Chromas Lite version 2.0². Sequences were aligned with reference genotypes to confirm positive identification for *H. contortus*, *T. circumcincta*, *Trichostrongylus axei*, *T.

---

colubriformis, C. ovina, O. venulosum or O. columbianum (GenBank AJ57746.1, AJ577463.1, AY439026.1, EF427624, AY439021.1, Y10790.1 and AJ006150, respectively) using Clustal W\(^3\).

2.7 Statistical analysis

WECs were adjusted for faecal consistency prior to statistical analysis. FCS was adjusted according to the following equation (Le Jambre et al., 2007):

\[
\text{Adjusted WEC} = \left( \frac{\text{Raw WEC}}{34.21 - 5.15 \times \text{FCS}} \right) \times 29.06
\]

Statistical analyses were performed using SPSS Statistics 17.0 for Windows. The WEC data were categorised as positive (WEC ≥50 epg) or negative (no strongylid eggs detected). To assess the level of agreement between the McMaster WEC and PCR tests, Cohen’s Kappa (κ) statistic was calculated at each sampling occasion, overall for each farm (all five samplings combined) and overall for the entire study (both farms combined). Categorical data were analysed to test the level of agreement between WEC and PCR results (positive vs. negative), with differences between PCR against WEC, along with WEC against PCR, both accounted for in calculation of the κ statistic.

Sampling prevalences (including 95% confidence intervals) were calculated using the exact binomial method for individual strongylid species, mixed strongylid infections (lambs PCR positive for at least two or more strongylid species at a sampling) and overall (lambs PCR positive for a strongylid species across all five samplings) (Thrusfield, 2007). The mean number of strongylids detected per lamb at each sampling was calculated using arithmetic means for each farm. Odds ratio risk analyses with Pearson’s \( \chi^2 \) test for independence or Fisher’s exact two-sided test for significance were conducted to determine if there were significant associations between PCR detection procedures for

\(^3\) See: http://www.clustalw.genome.jp.
Correlation between adjusted WEC and number of strongylid species detected per lamb was estimated by linear regression using a Pearson correlation two-tailed test for significance.

3. Results

3.1 PCR inhibition and spike analyses

PCR products were amplified from DNA extraction positive controls (faecal samples with WEC <50 epg spiked with L₃) with product sizes in accordance with those described for each strongylid species by Bott et al., (2009). These PCR products amplified in accordance with the L₃ tissue DNA extracts (PCR positive controls), while no products were amplified from DNA extraction negative controls (faeces only with WEC <50 epg) for any of the species-specific PCRs. Using spike analysis on different serial dilutions for each strongylid species, the minimum amount of genomic DNA required for successful PCR amplification was 2.0-5.0 pg (Table 3).

3.2 Comparison of worm egg counts with PCR

The levels of agreement between WEC and PCR results in identifying patent strongylid infections (κ statistic) are shown in Table 4. The overall κ value was 0.95 ± 0.01 (standard error).

Fourteen of 1004 samples (1.4%) were McMaster WEC flotation positive (all 50 epg) and PCR negative. Following spiking of these sample extracts with 1 μL purified DNA from each of the five strongylid species, the spiked DNA mixture was screened by each species-specific PCR to test for inhibition. All PCR assays amplified in accordance with L₃ tissue DNA extracts, indicating that no faecal inhibition was present.

Five of 1004 samples (0.5%) that were McMaster WEC negative and PCR positive were rescreened and the PCR products were sequenced. The sequenced products T.
circumcincta \((n = 1)\), T. colubriformis \((n = 2)\) and C. ovina \((n = 2)\) were 100% identical with
GenBank reference sequences, confirming the initial results.

3.3 Epidemiology of strongylid species

The overall strongylid prevalence was 97.2\% (95\% confidence interval, 95\% CI, 92.0-99.4) for Farm A and 94.1\% (95\% CI 88.3-97.6) for Farm B. Teladorsagia circumcincta was the most frequent strongylid species identified in both lamb flocks, followed by Trichostrongylus spp., C. ovina and Oesophagostomum spp. (Table 4). Patent H. contortus infections were not identified by PCR at either farm. No strongylid species were associated with an increased risk of non-pelleted faeces (FCS ≥3) in lambs from Farm A. T. circumcincta was the only species associated with an increased risk of non-pelleted faeces on Farm B, where lambs which tested T. circumcincta-positive, were 2.30 (95\% CI 1.19-5.20; \(P = 0.043\)) and 2.63 (95\% CI 1.23-7.14; \(P = 0.041\)) times more likely to have non-pelleted faeces than negative lambs at the second and final samplings, respectively.

The number of samplings that lambs tested positive for each strongylid species is presented in Figure 1. The arithmetic mean number of strongylid species detected per lamb ranged from 0.43 ± 0.06 (mean ± SE) to 2.19 ± 0.07 for Farm A and from 0.34 ± 0.05 to 1.22 ± 0.08 for Farm B at different times of sampling. Lambs (2-6 weeks of age) at the first sampling had the lowest mean number of strongylid species detected per lamb on both farms. The highest mean number of strongylid species detected per lamb was identified in both flocks, either at the sampling when an anthelminthic treatment was administered (Farm B) or at the final sampling with lambs in lairage awaiting slaughter (Farm A). For Farm A, the mean number of strongylid species detected per lamb at lairage, was significantly different to all other samplings \((P <0.001)\), while, for Farm B, the sampling when an anthelminthic treatment was administered was significantly different to
the first and third samplings ($P < 0.001$). Figure 2 shows the proportions of lambs from each farm with the relevant number of strongylid species detected per individual.

### 3.4 Mixed strongylid infections

In both flocks, 100% of lambs were PCR positive for two or more strongylid genera at one or more samplings, with mixed strongylid prevalences ranging from 26.6-100.0% (Table 4). For both farms, *T. circumcincta* and *Trichostrongylus spp.* were the most frequent mixed strongylid infections.

### 3.5 Faecal worm egg count correlations with PCR results

On both farms, the highest individual strongylid species prevalences, mixed strongylid prevalence and mean number of strongylid species detected per lamb coincided with the highest average flock WEC; 1,164 epg (adjusted WEC at the final sampling) for Farm A and 273 epg (adjusted WEC at the third sampling) for Farm B (Table 4). The number of strongylid species detected per lamb had a significant positive correlation with WEC and adjusted WEC ($r^2 0.026-0.591$; Table 4).

### 4. Discussion

This study utilised recently described diagnostic PCRs (Bott *et al.*, 2009), which had previously been used to screen genomic DNA extracted directly from faeces (Sweeny *et al.*, 2011). However, in contrast to the previous study, the sensitivity of such a diagnostic approach was examined over an extended period of 8 months (across five separate sampling occasions), to assess the sensitivity of the previously reported molecular diagnostic procedure in comparison with WEC. Bott *et al.*, (2009) demonstrated that PCRs detected naturally acquired strongylid infections following the separation of worm eggs from faeces by sodium nitrate flotation and column-purification. The present study demonstrated that DNA extractions performed directly on faeces, by the use of a commercial DNA isolation kit, could overcome possible inhibitory elements within faeces.
(collected from lambs both on-farm and in lairage) that may interfere with PCR amplification. This supported the recent findings of high levels of agreement (93%) between PCR and WEC results (Sweeny et al., 2011), with the current study highlighting a varying sensitivity range at different samplings (κ values from 0.85-1.00). All positive controls amplified at the correct product length and all sequenced PCR products were 100% identical with GenBank reference sequences. Although complete inhibition did not occur, the minimum amount of genomic DNA required for PCR amplification of the ITS-2 region, was higher (2-5 pg) in this study compared to a previous study using egg flotation and column-purification (0.1-2 pg) (Bott et al., 2009). Despite no minimum amount of genomic DNA for PCR amplification recorded in a previous study (Sweeny et al., 2011) which utilised the same molecular diagnostic procedure as the present study, the minimum DNA detection limit results suggest that low concentrations of faecal inhibitors were likely to exist following DNA extraction directly from faeces.

The source of strongylid DNA includes eggs that have been produced by mature female worms and passed in the faeces, i.e. established, patent infections. Screening for strongylid DNA extracted directly from faeces by PCR for such purposes has some disadvantages. The first is the inability to distinguish between patent and non-patent sources of strongylid DNA. Strongylid worm/larval tissue segments passed in faeces potentially can be amplified by PCR if the DNA yield is above the minimum detection limit. At the fourth sampling for Farm A lambs (6-7 months old) and the third sampling for Farm B lambs (3-4 months old), kappa values were at their lowest (89% and 85%, respectively). Furthermore, correlations between PCR and WEC decreased as the lambs aged (except for the final sampling on Farm B). This could be due to an increased probability of false PCR positive identification (non-patent DNA sources), whereby acquired immunity developed by lambs grazing strongylid contaminated pastures, would lead to a decreased establishment of ingested L₃ and greater biomass of larval tissues (non-patent DNA)
passed in the faeces (Dobson et al., 1990a; Dobson et al., 1990c, b, d). The higher levels of agreement observed in lairage (100% and 93% for Farms A and B, respectively) are potentially attributed to an increased worm egg concentration in faeces, as lambs were held off feed and had limited water access prior to slaughter (Jacobson et al., 2009).

Nevertheless, with some lambs testing negative for strongylid infections while grazing pastures infested with larvae, the strong correlations between WECs and the number of strongylid species detected per lamb by PCR and the highest strongylid species prevalences coinciding with the highest average flock WECs, all indicate that strongylid eggs are likely to be the main source of DNA produced from faecal extractions.

Commercial DNA isolation kits require only a small sub-sample of faeces to be taken from the centre of each sample. Uneven distribution of strongylid worm eggs within faeces (Sinniah, 1982; Tarazona, 1986; Hoste et al., 2001) provokes uncertainty as to whether this sub-sample (0.25-0.30 g) is representative and consistently able to provide an accurate representation of a faecal sample and to achieve an accurate diagnosis. The high levels of agreement from the two category analyses between the McMaster WECs and PCR tests suggest that, although a lower quantity of faeces was used in the molecular identification method, outcomes were similar to those using the McMaster WEC method.

Post-mortem examinations and total worm counts were not performed in the present study, so the infection status of individual lambs was unknown. False PCR positive identification of patent strongylid infections was reported to be low in a recent study where 100 sheep with a very low risk of strongylid infection risk (housed indoors and treated with anthelmintics) never tested PCR positive (Roeber et al., 2011). The 14 samples in this present study that were McMaster WEC positive and PCR negative, amplified in accordance to the species-specific PCRs when spiked with L₃ DNA. Intermittent shedding of strongylid eggs by small numbers of established female worms, an uneven distribution of strongylid eggs within faecal samples (Sinniah, 1982; Tarazona, 1986; Hoste et al.,
2001) or DNA concentrations below the minimum DNA detection limit for PCR (2-5 pg), may have lead to some strongylid infections going undetected. Such findings may also indicate the presence of a strongylid species not screened for by a species-specific PCR assay, which had eggs with an indistinguishable morphology from those of other species. For instance, Cooperia spp. occur at low proportions in worm populations in sheep flocks in southern Australia (Anderson, 1972, 1973; Barger and Southcott, 1975), but screening for this strongylid was not performed in the present study.

The main advantages of screening DNA extracted directly from faeces by PCR compared to larval culture, are an increased speed of diagnostic testing, unbiased detection and the ability to detect low worm burdens in small volumes of faeces from individual animals. Fast, accurate identification of strongylid species present within a flock would be useful for the rapid diagnosis of highly pathogenic strongylids (such as H. contortus), which can cause high mortalities in susceptible sheep (Dargie and Allonby, 1975; Abbott et al., 1986). This PCR technique determines the proportion of a flock which harbours specific strongylid species and would be beneficial in providing both a fast, accurate diagnosis for effective strongylid control programmes and also for monitoring treatment efficacy (Besier and Love, 2003; Coles et al., 2006; Woodgate and Besier, 2010). Species-specific diagnosis has important implications for treatment decisions, surveillance of anthelminthic resistance and where a high degree of precision for strongylid species identification is required. In contrast, larval cultures have a biased tendency to identify particular larval species (Dobson et al., 1992).

Haemonchus contortus was not identified on either farm, even though all spiked H. contortus positive controls were amplified successfully by PCR. Optimum survival of free-living H. contortus stages occur where adequate moisture, warm temperatures and green pastures enhance L₃ survival (Besier and Dunsmore, 1993a, b) and hot dry conditions dramatically reduce the survival of H. contortus larvae (Dobson and Barnes, 1995). The
inland regions of southern Western Australia, where the two farms in this study were located, are characterised by environmental conditions whereby high temperatures and a lack of moisture prevail over summer months. Such conditions are unfavourable for *H. contortus* larvae survival (Besier and Dunsmore, 1993a, b).

The high sensitivity of the molecular diagnostic procedure utilised to detect strongylid infections from lambs of various ages, is potentially superior to microscopy-based methods. However, when costs of running the test are included in decision making, affordable diagnostics are typically preferred (Hunt, 2011). Despite DNA polymerases incurring extra costs, PCRs offer increased sample throughput, decreased labour input and the potential to screen for a variety of livestock pathogens (parasites, bacteria and viruses). The major disadvantage of the molecular approach utilised in this study i.e. the inability to distinguish between patent and non-patent sources of strongylid DNA, is potentially an advantage for the early detection of strongylid parasites which contaminate pastures. The differentiation of DNA origin source would not be critical for such a procedure, although the ability to quantify the level of larvae exposure would be. There is potential for further investigation in early larvae detection on pastures, by use of a molecular procedure.

5. Conclusions

This study utilised PCR assays to identify different patent strongylid species infections in lambs, using DNA extracted directly from faeces with a commercial DNA isolation kit. The sensitivity of such a diagnostic approach was examined over an extended 8 month period across multiple sampling occasions, with levels of agreement between WEC and PCR results ranging from 85-100%. The DNA extraction positive controls amplified in accordance with *L₃* tissue DNA extracts. Serial dilutions revealed the minimum amount of extracted genomic DNA required for successful PCR amplification was 2.0-5.0
The highest strongylid species prevalences, mixed strongylid prevalences and mean number of strongylid species detected per lamb coincided with the highest average flock WECs on both farms. These findings indicate that strongylid eggs are likely to be the main source of DNA in faecal DNA extracts. Although this molecular technique offers the potential to identify different strongylid species infections, further comparisons against total worm eggs counts are required to more accurately determine the sensitivity and specificity of such a procedure, as it is potentially influenced by non-patent larvae tissues in faeces, as lambs acquire immunity to these parasites.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

We are grateful to the Australian Research Council (ARC) for funding this research and also to the farmers who cooperated with our research team for this study and aided our collection of faecal samples. A special thanks to Rob Shepherd (Hillside Tender Meats, Narrogin), along with Justin and Jason (Fletchers International, Narrikup) for their advice and assistance with collection of samples and data at the abattoirs. A final special thanks to Aileen Elliot (Murdoch University) for her microscopy training and assistance with culturing strongylid L₃ for tissue DNA extraction to use as PCR positive controls.

References


Table 1

Faecal sampling occasions, lamb age and day of study.

<table>
<thead>
<tr>
<th>Faecal sampling occasion</th>
<th>Stage in lamb's life</th>
<th>Lamb age</th>
<th>Study day</th>
<th>Date</th>
<th>Farm A</th>
<th>Study day</th>
<th>Date</th>
<th>Farm B</th>
<th>Lambs sampled (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling 1</td>
<td>Marking</td>
<td>2-6 weeks</td>
<td>0</td>
<td>July 23rd 2009</td>
<td>111</td>
<td>0</td>
<td>27 August 2009</td>
<td>124</td>
<td></td>
</tr>
<tr>
<td>Sampling 2</td>
<td>Pre-weaning I</td>
<td>2 months</td>
<td>39*</td>
<td>Sept 1st 2009</td>
<td>109</td>
<td>42</td>
<td>8 October 2009</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Sampling 3</td>
<td>Pre-weaning II</td>
<td>3-4 months</td>
<td>80</td>
<td>Oct 13th 2009</td>
<td>108</td>
<td>73*</td>
<td>8 November 2009</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Sampling 4</td>
<td>Post-weaning</td>
<td>6-7 months</td>
<td>194</td>
<td>Feb 8th 2010</td>
<td>109</td>
<td>181</td>
<td>24 February 2010</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>Sampling 5</td>
<td>Lairage</td>
<td>7-8 months</td>
<td>199/240</td>
<td>Feb 10th 2010</td>
<td>107</td>
<td>188</td>
<td>3 March 2010</td>
<td>119</td>
<td></td>
</tr>
</tbody>
</table>

Mar 23rd 2010

* Indicates after this sampling occasion that lambs received an anthelmintic treatment.
Table 2

Stepwise procedures, DNA extraction kits and purpose of the different methods utilised to extract genomic DNA from strongylid L₃ and faecal samples.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Methodology prior to DNA extractions</th>
<th>DNA extraction kit</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR positive controls</td>
<td><em>T. circumcincta</em>, <em>T. colubriformis</em>, <em>H. contortus</em>, <em>C. ovina</em> and <em>Oesophagostomum venulosum</em> L₃ were collected from larval cultures of fresh sheep faeces after microscopic identification. The different L₃ species were separated into 200 µL suspensions (each containing 100 L₃ of only one strongylid species).</td>
<td>DNeasy Blood and Tissue Kit (Qiagen).</td>
<td>Confirm that PCR assays and conditions achieve successful product amplification.</td>
</tr>
<tr>
<td>DNA extraction negative and positive controls</td>
<td>DNA was extracted from negative DNA extraction control samples; 250-300mg of faeces from a sample that returned three WECs &lt;50 epg; (n = 96) and positive DNA extraction controls; 250-300mg of faeces from a sample that returned three WECs &lt;50 epg and spiked with 100 µL (n = 100 L₃) suspension containing equal proportions of L₃ from all five strongylid species; (n = 96).</td>
<td>Power Soil DNA Isolation Kit (MO BIO Laboratories).</td>
<td>Used to determine whether the PCRs were capable of detecting strongylid DNA, whether cross contamination between samples occurred and if any faecal inhibitors were detected in samples.</td>
</tr>
<tr>
<td>Spike and serially diluted genomic DNA</td>
<td>DNA was extracted using 250-300 mg of faeces from a faecal sample that returned three WECs &lt;50 epg. Prior to the extraction each sample was spiked with 100 µL L₃ suspension (n = 100 L₃) containing whole L₃ from only one strongylid species. DNA concentrations were determined using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Genomic DNA was serially diluted from 10⁵ pg to 10,000, 1000, 100, 50, 10, 5, 2, 1 and 0.1 pg/µL. amplification length for each species-specific PCR.</td>
<td>Power Soil DNA Isolation Kit (MO BIO Laboratories).</td>
<td>To determine the minimum amount of genomic DNA extracted from faeces that would achieve successful PCR amplification.</td>
</tr>
<tr>
<td>Field faecal samples</td>
<td>Genomic DNA was extracted from 250-300 mg for each of the 1155 faecal samples collected from the identified lambs in this study.</td>
<td>Power Soil DNA Isolation Kit (MO BIO Laboratories).</td>
<td>Assess the sensitivity and capability of PCRs detecting strongylid infections across multiple samplings, to determine the levels of agreement between WEC and PCR.</td>
</tr>
</tbody>
</table>
Table 3

Minimum amount of genomic DNA extracted directly from faeces, which specific amplification of the ITS-2 region could be achieved using the individual primer sets shown in parenthesis.

<table>
<thead>
<tr>
<th>Farming property and parasite</th>
<th>Detection limit (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemonchus contortus (HAE-NC2)</td>
<td>2.0</td>
</tr>
<tr>
<td>Teladorsagia circumcincta (TEL-NC2)</td>
<td>5.0</td>
</tr>
<tr>
<td>Trichostrongylus spp. (TRI-NC2)</td>
<td>2.0</td>
</tr>
<tr>
<td>Chabertia ovina (CHO-NC2)</td>
<td>2.0</td>
</tr>
<tr>
<td>Oesophagostomum spp. (TRI-NC2)</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Table 4

Strongylid species prevalences and 95% confidence intervals in meat lambs on two farms in southern Western Australia.

<table>
<thead>
<tr>
<th>Sheep age</th>
<th>Farm A</th>
<th>Farm B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-weaning</td>
<td>Post-weaning</td>
</tr>
<tr>
<td></td>
<td>2-6 weeks</td>
<td>2 months</td>
</tr>
<tr>
<td>Day of study</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>Flock size</td>
<td>550</td>
<td>550</td>
</tr>
<tr>
<td>Stocking rate (DSE/ha)</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Identified lambs sampled (n)</td>
<td>111</td>
<td>109</td>
</tr>
<tr>
<td>Teladorsagia circumcincta prevalence</td>
<td>(14.4, 30.4)</td>
<td>(57.3, 75.7)</td>
</tr>
<tr>
<td>Trichostrongylus spp. prevalence</td>
<td>(5.7, 18.1)</td>
<td>(42.5, 61.9)</td>
</tr>
<tr>
<td>Chabertia ovina prevalence</td>
<td>(3.2, 13.7)</td>
<td>(24.3, 42.7)</td>
</tr>
<tr>
<td>Oesophagostomum spp. prevalence</td>
<td>(1.0, 9.0)</td>
<td>(3.2, 14.0)</td>
</tr>
<tr>
<td>Mixed strongylid prevalence</td>
<td>(25.5, 43.8)</td>
<td>(79.4, 92.8)</td>
</tr>
<tr>
<td>Average WEC ± SEM (epg)</td>
<td>29 ± 10</td>
<td>446 ± 45</td>
</tr>
<tr>
<td>Adjusted average WEC ± SEM (epg)</td>
<td>32 ± 11</td>
<td>552 ± 59</td>
</tr>
<tr>
<td>κ statistic ± SE</td>
<td>1.00 ± 0.08</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>Linear regression correlation ר² (P value) between the number of strongylid species per lamb and adjusted WEC</td>
<td>0.412</td>
<td>0.302</td>
</tr>
</tbody>
</table>

*a* Indicates that lambs received an anthelmintic treatment after this sampling.

*Not all samples had enough faecal material to conduct WEC.*

*Mixed strongylid prevalence: Lamb PCR positive for two or more strongylid species (Note: *Haemonchus contortus* was not identified in any lamb faecal samples).*

*SEM, Standard error of the mean; WEC, Worm egg count; epg, Eggs per gram.*

*Kappa statistic, The level of agreement between the PCR assays and McMaster WEC diagnostic tests for identifying patent strongylid nematode infections.*

| Stocking rate (DSE/ha) | 12 | 12 | 12 | 10 | 10 | 10 | 9 | 9 |

536  
537  
538  
539  
540  
541  
542  
543
Figure legends

**Figure 1.** The number of positive samplings that lambs tested positive for each strongylid species at two farms in Western Australia. A total of 107 and 119 lambs were sampled at all five samplings for Farm A and B, respectively.

**Figure 2.** Proportions of lambs with different numbers of strongylid species detected by PCR for each farm located in southern Western Australia.
Figure 1

This figure shows a bar chart that compares the number of lambs for different species across two farms (A and B). The y-axis represents the number of lambs (n), while the x-axis represents the number of positive samplings. The species represented are Teladorsagia circumcincta, Trichostrongylus spp., Chabertia ovina, and Oesophagostomum spp.

For Farm A, the chart indicates that Teladorsagia circumcincta and Trichostrongylus spp. have the highest number of positive samplings at 120 and 100, respectively. Chabertia ovina and Oesophagostomum spp. have lower counts.

For Farm B, the chart shows a similar trend with Teladorsagia circumcincta having the highest number of positive samplings at 120. Trichostrongylus spp. and Chabertia ovina have lower counts, while Oesophagostomum spp. shows the least positive samplings.

The bars for each farm are color-coded to distinguish between the species.
Figure 2

Number of strongylid species detected per lamb (n)

Proportion of lambs (%)

Sampling occasion
Farm A

0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%

1 2 3 4 5

Sampling occasion
Farm B

1 2 3 4 5

0 1 2 3 4