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A combined microscopic-molecular method for the diagnosis of strongylid infections in sheep

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ABSTRACT

We evaluated a combined microscopic-molecular approach for the diagnosis of key strongylid infections in sheep using panels of well-defined control and test samples. The method established is based on the separation of nematode eggs from faecal samples using a salt flotation procedure, the extraction and column-purification of genomic DNA, followed by real-time PCR and melting-curve analysis. Specific and semi-quantitative amplification from (a minimum of 0.1–2.0 pg) genomic DNA of *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp., *Cooperia oncophora*, *Oesophagostomum columbianum*, *Oesophagostomum venulosum* or *Chabertia ovina* is achieved using a specific, forward oligonucleotide primer located in the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA (rDNA) together with a conserved reverse primer in the large subunit of rDNA. Using a panel of well-defined genomic DNA samples from eggs from sheep monospecifically infected with *H. contortus* or *Te. circumcincta*, there was a correlation between cycle threshold (Ct) values in the PCR and numbers of egg per gram of faeces, thus allowing the semi-quantitation of parasite DNA in faeces. The findings of the present study indicate that a microscopic-molecular approach provides a useful tool for diagnosis, for epidemiological and ecological surveys as well as for integration into parasite monitoring, drug resistance (i.e. 'egg count reduction') testing or control programmes, particularly following semi- or full-automation.

1. Introduction

Parasites of livestock and other animals cause diseases of major socio-economic importance worldwide. The current financial losses to agriculture due to parasites have a substantial impact on farm profitability. For example, the annual cost associated with parasitic diseases in sheep and cattle in Australia has been estimated at ~1 billion Australian dollars (McLeod, 1995; Sackett and Holmes, 2006). Thus, there are major economic gains to be made by enhancing the control of important parasitic diseases. Nematode parasites of livestock are controlled predominantly by using chemotherapeutic agents (=anthelmintics). Even with strategic treatment, this type of control is costly and not always effective. In addition, the excessive use of anthelmintics has resulted in substantial and widespread problems with genetic resistance in nematode populations (Wolstenholme et al., 2004; Kaplan, 2004; Gilleard, 2006). Therefore, there is a need for the development of improved means of controlling parasitic nematodes.

The accurate diagnosis of parasitic diseases and drug resistance is central to epidemiology and control. Traditional parasitological approaches (see Bowman, 2009) , which are still routinely used, can be time-consuming to perform. For instance, using coproscopic methods, it is not always possible to identify or distinguish among individual eggs or larvae of different species of strongylid nematodes with a degree of certainty. In particular, the method of larval culture is laborious, takes 1 week to perform and can be inaccurate (cf. Dobson et al., 2002). Using this technique, the microscopic identification of L3s to genus or species requires experience but may still be unreliable. Therefore, an alternative approach is required.

DNA technology might provide avenues to overcome these limitations and to assist in developing improved diagnostic methods for strongylid nematodes of domestic animals. To date, a number of PCR assays has been developed for the identification or differentiation of strongylid eggs or larvae, utilising genetic markers in the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) or external transcribed spacer (ETS) of nuclear ribosomal DNA (rDNA). For instance, Zarlenga et al. (1998, 2001) developed sensitive PCR-based assays for the specific identification/differentiation and semi-quantitation of eggs for a number of economically

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important gastrointestinal strongylids of cattle. Subsequently, Schnieder et al. (1999) reported a genus-specific PCR for the differentiation of eggs or larvae of selected nematodes of ruminants. Extending this work, von Samson-Himmelstjerna et al. (2002) and Siedek et al. (2006) evaluated real-time PCRs, using genus-specific primer–probe combinations to ITS-2, for the quantitation of genomic DNA from L1s or L3s representing four trichostrongylid genera derived from larval cultures from faeces from hosts with monospecific or naturally acquired infections. While useful, these assays did not achieve species-specific differentiation or quantitation. Recently, in a 'proof-of-concept' study, Harmon et al. (2007) developed a quantitative, real-time PCR, based on ITS-2, for the quantification of *Haemonchus contortus* eggs and emphasised the limiting factors (e.g., effect of inhibitors, 'competing' and 'non-competing' DNA and embryonic development within the egg) that could impact negatively on the development of a multiplex PCR assay using this locus. In spite of these developments, no real-time PCR assay evaluated to date achieves species-specific diagnosis of strongylid infections in sheep.

In the present study, we evaluated a combined microscopic-molecular approach, aimed at the specific diagnosis of key strongylid infections in sheep under Australasian conditions. This technique is based on the separation of nematode eggs from faecal samples, the purification of genomic DNA, followed by real-time PCR of ITS-2 regions and melting-curve analysis. Specific and semi-quantitative amplification from picogram amounts of genomic DNA of *H. contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp., *Cooperia oncophora*, *Oesophagostomum columbianum*, *Oesophagostomum venulosum* or *Chabertia ovina* was achieved using a species- or genus-specific, forward oligonucleotide primer located in ITS-2 together with a conserved reverse primer in the large subunit of rDNA.

2. Materials and methods

2.1. Parasites and isolation of genomic DNA

Adult (usually male) strongylids representing 13 species were collected at necropsy from the gastrointestinal tracts of sheep, washed extensively in physiological saline and identified according to existing keys and descriptions (Skrjabin et al., 1991) (Table 1). Some species of trichostrongylid were produced in experimental sheep, raised and maintained under helminth-free conditions (see Campbell et al., 2008). The anterior and posterior ends of each nematode were cut off, cleared in lactophenol and preserved in polyvinyl lactophenol as a permanent mount for reference. Adults of *Dictyocaulus filaria*, *Protostrongylus rufescens* (Metastrongyloidea), *Trichuris ovis* (Trichuroidea), *Moniezia expansa* (Cestoda), *Fasciola hepatica*, *Calicophoron calicophorum* and *Paramphistomum ichikawai* (Digenea) as well as oocysts or cysts of *Eimeria ahsata*, *Cryptosporidium parvum* and *Giardia duodenalis*/*intestinalis* (Protozoa) (see Table 1) were also collected, washed extensively and identified morphologically or molecularly using established methods (Skrjabin et al., 1991; Monis et al., 1996; Jex et al., 2007; Bowman, 2009). All samples were frozen at −20 °C or −70 °C.

For the isolation of genomic DNA, parasites (usually individual parasites or eggs; packed volume of 50–100 μl) were thawed and placed in a 1.5 ml Eppendorf tube containing 100–200 μl of DNA-extraction buffer (20 mM Tris–HCl, pH 8, 50 mM EDTA, 1% w/v SDS plus 0.5 μg/μl proteinase K) and incubated at 37 °C for 12–14 h. Genomic DNA was also isolated from faeces or liver tissue from a helminth-free sheep. A packed volume of 50–100 μl (faeces, diced tissue or packed cells) was transferred to a tube, suspended in extraction buffer and centrifuged at 12,000g; genomic DNA was purified from the supernatant using a mini-spin column (Wizard™ DNA Clean-Up), according to the manufacturer's instructions. For long-term storage (months to years) at −70 °C, genomic DNA samples were transferred to 0.6 ml double-lock PCR tubes.

2.2. Conventional coprological methods

Fresh faecal samples from individual or groups of sheep were collected from various geographical regions in Victoria, Australia. The samples were chilled (4–10 °C) after collection, transported to the laboratory and immediately (within 8–10 h) subjected to faecal flotation (MAFF, 1977) for the enumeration of strongylid nematode eggs (see Section 2.3); the parasitological findings and relevant epi-

Table 1
Genomic DNA samples used in this study.

Description	Group	Species	Host species	Site	Sample code	Geographical origin[a]
Test samples	Gastrointestinal strongylids	*Haemonchus contortus*	Sheep	Abomasum	HcNb5	Victoria
				Abomasum	Hc33	Victoria
		Teladorsagia circumcincta	Sheep	Abomasum	OcNB1.2	Victoria
		Trichostrongylus axei	Sheep	Abomasum	Ta5	Victoria
		Trichostrongylus colubriformis	Sheep	Small intestine	Tc14	France
		Trichostrongylus rugatus	Sheep	Small intestine	Tru2	Victoria
		Trichostrongylus vitrinus	Sheep	Small intestine	Tv2	South Australia
		Cooperia oncophora	Sheep	Small intestine	Co9	Victoria
		Chabertia ovina	Sheep	Large intestine	ChoNB5	New South Wales
		Oesophagostomum columbianum	Sheep	Large intestine	OecNb4	New South Wales
		Oesophagostomum venulosum	Sheep	Large intestine	OevNB22	Victoria
Control samples	Gastrointestinal strongylids	*Nematodirus filicollis*	Sheep	Small intestine	Nf3	Germany
		Nematodirus helvetianus	Sheep	Small intestine	Nh17	France
		Nematodirus spathiger	Sheep	Small intestine	Ns4	Germany
	Lungworms	*Dictyocaulus filaria*	Sheep	Lungs	DfNB1.7	Victoria
		Protostrongylus rufescens	Sheep	Lungs	Pr2	Victoria
	Whipworm	*Trichuris ovis*	Sheep	Large intestine	Tro6	USA
	Liver fluke	*Fasciola hepatica*	Sheep	Liver/bile ducts	Fh2	Victoria
	Paramphistomes	*Calicophoron calicophorum*	Sheep	Rumen/reticulum	Cc1	New South Wales
		Paramphistomum ichikawai	Cattle	Rumen/reticulum	Pi1	New South Wales
	Tapeworm	*Moniezia expansa*	Sheep	Small intestine	M8	Victoria
	Protozoa	*Eimeria ahsata*	Sheep	Small intestine	Eah2	Canada
		Cryptosporidium parvum	Cattle	Small intestine	SC26	Switzerland
		Giardia duodenalis	*In vitro* culture	Small intestine	Ad23	South Australia
	Host tissue	Sheep	–	Liver	–	Victoria

[a] States of Australia, if not otherwise stated

demiological information were entered directly into a spreadsheet. A proportion of each faecal sample was stored at −20 °C.

2.3. Isolation/purification of genomic DNA from strongylid eggs, and the enumeration of eggs in faecal samples from sheep

Genomic DNA was isolated from strongylid eggs from fresh faecal samples using the following procedure: (i) 4 g of faeces were homogenised in 60 ml of saturated sodium nitrate (specific gravity: 1.3), sieved (~1 mm mesh size) and transferred into a 50 ml conical tube (Falcon); (ii) a total of 1.5 ml of the suspension was aspirated using a sieve-top pipette and three chambers of a McMaster chamber were each filled with 0.3 ml; strongylid eggs were counted in each chamber and the mean number of eggs per gram (EPG) calculated; (iii) subsequently, the 50 ml tube was centrifuged at 1000*g* for 10 min, and 5 ml of supernatant (containing the eggs) were transferred to a fresh 50 ml tube and diluted with 45 ml of H_2O; (iv) after centrifugation (1000*g* for 10 min), the supernatant was removed and the pellet washed once more in the same manner; (v) the final pellet was resuspended in 500 µl H_2O and 100 µl glass beads (~1 mm in diameter, Sigma), 1% SDS and 500 µg/ml proteinase K (Boehringer) were added; (vii) this suspension was incubated (in an orbital shaker) for 15 h at 37 °C; (viii) the suspension was centrifuged at 3200*g* for 10 min; (ix) the supernatant was transferred to a 1.5 ml Eppendorf tube and centrifuged at 12,000*g* for 5 min; (ix) total genomic DNA was purified using a minicolumn (Wizard™ DNA Clean-Up, Promega) and eluted in 40 µl H_2O; (x) individual samples were diluted 1/50, and then kept at 4 °C until molecular analysis (same day) or stored at −70 °C for longer periods. In addition to the latter approach, genomic DNA was isolated directly from selected faecal samples (n = 53) using the C-Qentec™ commercial service provided by the South Australian Research and Development Institute (SARDI), Adelaide, South Australia. Some of these samples were from helminth-naïve sheep, raised indoors to ensure that they were free from parasitic nematodes.

2.4. Design of oligonucleotide primers to the ITS-2 sequences of key parasites of sheep

Specific primers were designed to internal regions of ITS-2 for key genera or species of strongylid nematodes (see Table 1), selected based on their pathogenicity and/or economic impact in a range of countries, including Australia. The ITS-2 sequences of *H. contortus* (GenBank Accession No. X78803; Stevenson et al., 1995), *Te. circumcincta* (Accession No. X86026; Stevenson et al., 1996), *Trichostrongylus axei*, *Tr. colubriformis*, *Tr. vitrinus*, *Tr. rugatus* (Accession Nos. X78065, X78063, X78064 and Y14818; Hoste et al., 1995; Chilton et al., 1998), *Co. oncophora* (Accession No. X83561; Newton et al., 1998a), *N. filicollis*, *N. helvetianus*, *N. spathiger* (Accession Nos. Y14010–Y14013; Newton et al., 1998b), *Oe. venulosum* (Accession No. Y10790; Chilton et al., 1997; Newton et al., 1998c) and *Ch. ovina* (Accession No. Y10789; Chilton et al., 1997) were aligned manually and then adjusted according to the predicted secondary structure for the precursor rRNA (see Chilton et al., 1998; Newton et al., 1998a,b,c), in order to increase positional similarity in regions with a greater level of nucleotide difference among species (see Fig. 1). Then, primers were designed following the criteria used by Hung et al. (1999), considering the principles for allele-specific amplification, which utilises the refractoriness of primers with 3′-end mismatch(es) in the PCR to achieve selective amplification (e.g., Matsunaga et al., 1995; Rhodes et al., 1997). The locations of the primers designed specifically to the ITS-2 sequences of key nematodes are shown in Fig. 1.

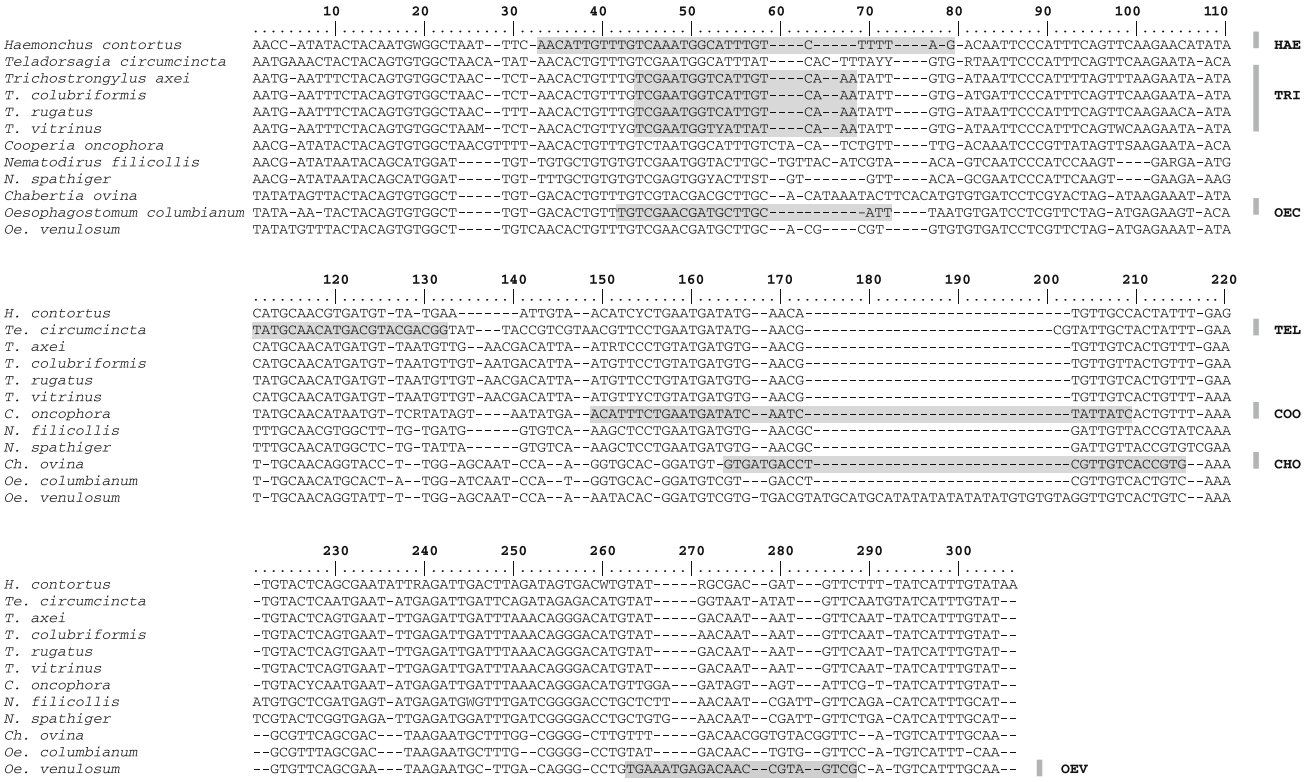

Fig. 1. Alignment of 5.8S, ITS-2 and partial 28S nuclear ribosomal DNA sequences, and location (grey) of individual (forward) primers (HAE, TEL, TRI, COO, OEC, OEV and CHO), designed to the ITS-2 and evaluated in silico for specificity. Primer NC2 (reverse) is located in the 5′-region of the 28S rRNA gene. Polymorphic positions indicated with International Union of Pure and Applied Chemistry (IUPAC) codes.

Table 2
Specific forward primers, used together with reverse primer NC2, and their characteristics (including sequence, G+C content, melting temperature as well as amplicon length following PCR).

Species	Primer	Primer sequence (5′–3′)	Start position in ITS-2[a]	GC (%)	Melting temperature (°C)	Amplicon length (bp)
Haemonchus contortus	HAE	CAAATGGCATTTGTCTTTTAG	41	33	47	265
Teladorsagia circumcincta	TEL	TATGCAACATGACGTACGACGG	98	50	55	218
Trichostrongylus spp.	TRI	TCGAATGGTCATTGTCAA	40	37	45	267–268
Cooperia oncophora	COO	CATTTCTGAATGATATCAATCTATTATC	131	25	51	179
Chabertia ovina	CHO	GATGACCTCGTTGTCACCGTG	143	57	56	162
Oesophagostomum columbianum	OEC	TGTCGAACGATGCTTGCTTT	34	45	50	251
Oesophagostomum venulosum	OEV	TGAAATGAGACAACCGTAGTCG	222	45	53	105

[a] Cf. Fig. 1.

Their characteristics (including G+C contents and melting temperatures) are listed in Table 2.

2.5. Conventional and real-time PCR and melting-curve analysis

Regions of nuclear rDNA were amplified by PCR using seven different primer pairs in separate reactions (see Fig. 1). Precautions were taken to prevent contamination at every step of the procedure. PCR conditions were optimised by titration of $MgCl_2$ and dNTP amounts, as well as varying annealing temperatures, cycle numbers and step times. PCRs (50 μl) were performed in 10 mM Tris–HCl, pH 8.4; 50 mM KCl; 3.5 mM $MgCl_2$; 0.1% Triton X-100; 250 μM of each dNTP; 50 pmol of each primer with 1 U GoTaq polymerase (Promega). Two microlitres of genomic template (typically 5–100 ng) were added to individual reactions. Samples without genomic DNA (no-DNA controls) and a known positive (genomic DNA from *H. contortus*) control were included in each PCR run. Also, host DNA was subjected to the same amplification procedure as parasite DNA.

For the assessment of primer specificity, cycling was conducted in a conventional thermal cycler (480, Perkin Elmer Cetus) using the following parameters: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and 72 °C for 30 s (extension), followed by a final extension at 72 °C for 7 min. Amplicons were resolved on 2% agarose-TBE (65 mM Tris–HCl, 27 mM boric acid, 1 mM EDTA, pH 9) gels, stained with ethidium bromide and detected upon ultraviolet transillumination using an electronic documentation system (Gel-Doc, Bio-Rad). Subsequently, the specificity of amplicons was verified by automated DNA sequencing (using BigDye chemistry, ABI). To do this, amplicons were column-purified (using Wizard™ PCR-Preps, Promega) and sequenced directly using primer NC2. The identity of each (5′) sequence tag was established based on comparisons with reference sequences from key strongylid nematodes available in the GenBank database.

Real-time PCR was performed in a Rotor-Gene thermal cycler (RG6000, 65HO, Corbett Research Pty Ltd.) using the same cycling conditions as for the conventional PCR, except that 8 μM of SYTO® 9 (Invitrogen) was used in each reaction and different polymerases (i.e. GoTaq [Promega] and a "hot-start" polymerase provided in the Sensimix dT kit [Quantace]) were employed at different stages of the evaluation of the PCR (typically using 5–100 ng of genomic DNA). SYTO® 9 was used as the dye, because it does not inhibit the PCR and because of its increased stability compared with other dyes (Monis et al., 2005a; Gudnason et al., 2007). Optical measurements in the green channel (excitation at 470 nm and detection at 510 nm) were recorded at the end of each extension step. Amplicons (duplicates) were subjected to melting-curve analysis by increasing the temperature from 75 to 85 °C at 0.1 °C per s, recording the changes in fluorescence, using Rotor-Gene v.1.7.87 software, and applying the normalisation option and a confidence threshold of 96%.

3. Results

3.1. Assessment of the specificity of primers in the conventional PCR

Following the theoretical design of forward primers (see Fig. 1 and Table 2), their sequences were subjected to BLASTn analysis (at http://blast.ncbi.nlm.nih.giv/Blast.cgi) to verify their specificity in silico. Each primer matched exclusively the sequence from which it was designed. The seven primer pairs, HAE–NC2, TEL–NC2, TRI–NC2, COO–NC2, OEC–NC2, OEV–NC2 and CHO–NC2 (Fig. 2), were then evaluated for their specificity in the PCR. Amplicons were examined by agarose gel electrophoresis and sequenced. These primer pairs were tested individually in the PCR using genomic DNA samples from individual species of strongylid included (see Table 1). The primer pair NC1–NC2 was used to control for inhibition in, and amplification efficiency from, individual genomic DNA samples (Fig. 2). For all other primer pairs, specific amplification was achieved from the appropriate genus or species, and individual amplicons were of the expected sizes (Fig. 2). In addition to the products (267 or 268 bp) amplified specifically by PCR from genomic DNA of each of the four *Trichostrongylus* species using primer pair TRI–NC2, a smaller, much fainter product (160 bp) was amplified from each *Oe. columbianum* and *Oe. venulosum* using this primer pair and GoTaq polymerase (Fig. 2). While a specific product of 251 bp was amplified from genomic DNA of *Oe. columbianum* in the PCR using the primer pair OEC–NC2, a very faint, non-specific product of ~280 bp was amplified from genomic DNA of *Pa. ichikawai* (paramphistome) using the same primer pair and GoTaq polymerase.

The direct sequencing (employing the primer NC2) of all amplicons from strongylid nematodes, produced using each of the seven primer pairs (see Table 2), confirmed that each product was derived from the appropriate rDNA region and species (cf. Figs. 1 and 2). The faint 160 bp product amplified from *Oe. columbianum* or *Oe. venulosum* in the PCR using primer pair TRI–NC2 was demonstrated (by sequencing) to represent a specific sequence tag within ITS-2 of either of these two species (starting at position 233; see Fig. 1). The specificity of each primer pair was also assessed in the PCR by cycling with a range of (heterologous) genomic DNAs (~10 ng per PCR) from each *D. filaria, Pr. rufescens*, *Tr. ovis, M. expansa*, *F. hepatica*, *Ca. calicophorum*, *Pa. ichikawai*, *E. ahsata*, *Cr. parvum* and *G. intestinalis,* sheep liver and 'helminth-free' faecal samples. Except for the faint ~280 bp product amplified from *Pa. ichikawai* using the primer pair OEC–NC2, no amplicons were produced from any of the other control samples using each of the seven "specific" primer pairs (Fig. 2). This ~280 bp product could not be sequenced using either OEC or NC2, indicating its non-specificity.

Subsequently, the 'sensitivity' of the PCR using each of the different primer pairs was determined by amplification from serial titrations of genomic DNA from homologous species. The smallest amount of genomic DNA from which effective PCR amplification

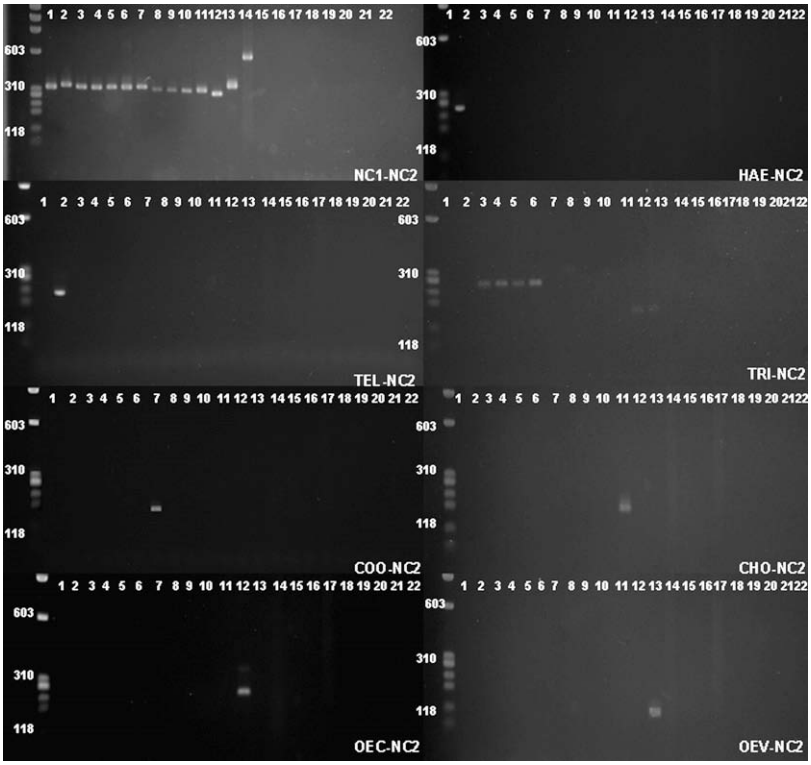

Fig. 2. Representative results demonstrating the specificity of individual primer pairs in PCR. Agarose gels displaying amplicons produced by conventional PCR using primer pairs NC1–NC2, HAE–NC2, TEL–NC2, TRI–NC2, COO–NC2, CHO–NC2, OEC–NC2 and OEV–NC2 (cf. Fig. 1). Genomic DNA samples from adult males of *Haemonchus contortus, Teladorsagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus*, *Trichostrongylus rugatus*, *Cooperia oncophora*, *N. filicollis*, *N. spathiger*, *Oesophagostomum columbianum*, *Oesophagostomum venulosum* and *Chabertia ovina* were included (lanes 1–13). Control samples from other parasites, including the helminths *Dictyocaulus filaria, Protostrongylus rufescens*, *Trichuris ovis, Fasciola hepatica* and the protozoa *Cryptosporidium parvum* and *Giardia duodenalis/intestinalis* (lanes 14–19, respectively), as well as sheep liver, and 'parasite-free' faecal samples (lanes 20–21, respectively), and a no-DNA control (lane 22) were also included. The primer pair NC1–NC2 was used to control for inhibition in the PCR and amplification efficiency from individual DNA samples. The specificity of individual amplicons produced using each of the eight primer pairs was verified by direct sequencing.

could be achieved, with amplicons being detected on agarose gels, was 0.1–2 pg for each of the seven primer pairs (Table 3). When excess genomic DNA from all heterologous species of strongylids (10 ng for each species) was added to a minimum amount (0.1–2 pg) of template representing the species being amplified with its specific primer pair, no reduction in the 'sensitivity' or amplification efficiency of the PCR was detected; the same applied when the same amount of genomic DNA from all of the non-strongylid parasites or host-DNA control (equal amounts; ~5–10 ng of each) was added to the template (results not shown).

3.2. Evaluation of real-time PCR-coupled with melting-curve analysis

Following the assessments of the specificity, 'sensitivity' and amplification efficiency for each primer pair in the conventional PCR, the real-time PCR was evaluated and then employed routinely. No difference in the performance of the latter PCR was observed, except that the smallest amount of genomic DNA from which effective real-time PCR amplification (using individual primer pairs) was achieved was 0.1–1 pg (in the presence or absence of heterologous DNA) (see Table 3).

Using serial titrations of genomic DNA (0.1, 1, 10, 100 and 1000 pg), the kinetics of the real-time PCR using GoTaq (Fig. 3) and the melting-curves of amplicons were determined (individual curves available upon request). Amplicons produced on distinct days were subjected (in triplicate) to melting-curve analysis on three different days. Amplification and melting-curves for individual species were reproducible on distinct days for amplicons produced at different time points using varying amounts of starting template in the PCR. The mean peak melting temperatures (in °C) for amplicons produced by PCR (employing GoTaq polymerase) using six of the seven specific primer pairs were ~80.8 (±0.13) (HAE–NC2), 81.0 (±0.20) (TEL–NC2), 80.9 (±0.30) (COO–NC2), 85.5 (±0.19) (OEC–NC2), 82.5 (±0.17) (OEV–NC2) and 85.4 (±0.12) (CHO–NC2). Given that the primer pair TRI–NC2 amplified specific products from ITS-2 of each of four species of *Trichostrongylus* included, individual species were tested separately (as previously) to establish the characteristic melting-curve for each species. The mean peak melting temperatures (in °C) for amplicons from *Tr. axei*, *Tr. colubriformis*, *Tr. rugatus* and *Tr. vitrinus* were 80.7 (±0.19), 80.31 (±0.06), 80.9 (±0.09) and 80.5 (+0.09), respectively. The mean peak melting temperatures (83.7 ± 0.3 and 83.35 ± 0.4) of the smaller, much fainter product (160 bp) amplified from *Oe. columbianum* and *Oe. venulosum* (see Section 3.1) differed by 3–3.5 °C from those amplified from individual species of *Trichostrongylus* using primer pair TRI–NC2 (not shown). When equal amounts (10 ng/μl) of *Tr. colubriformis, Oe. columbianum* and *Oe. venulosum* genomic DNA were mixed and PCR amplified using the primer pair TRI–NC2, the melting-curve obtained was consistent with that of *Tr. colubriformis*. The mean peak melting temperature (79.9 ± 0.08) of the ~280 bp product amplified from *Pa. ichikawai* using primer pair OEC–NC2 (see Section 3.1) was consistently ~5 °C lower than the peak for the 251 bp product amplified from *Oe. columbianum* using primer pair OEC–NC2 (not shown). Normalised melting-curve data provided confidence regarding the identity of the amplicons; this was evidenced, in particular, by the ability of these curves to delineate among the four species of *Trichostrongylus* included. Since melting-curve analysis is a

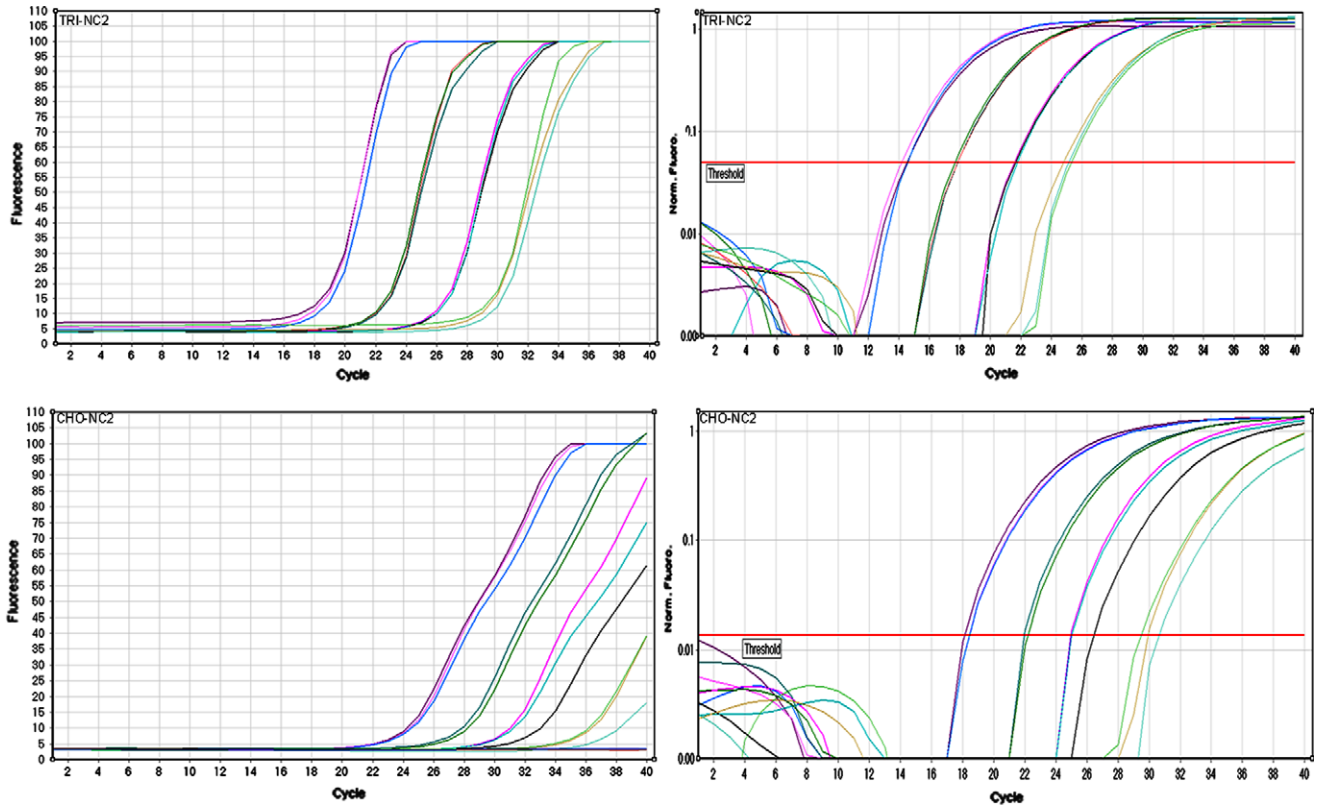

Fig. 3. Graphic display of the kinetics of the real-time PCR (using GoTaq), indicating cycle threshold (Ct) values for selected species, as examples.

mutation detection approach (Reed et al., 2007) and has the capacity to detect point mutations between amplicons (typically 70–250 bp in size), the definition of the melting-curves for the specific amplicons from individual species provided the basis for an independent confirmation of the identity of each amplicon (and its sequence) following real-time PCR amplification (see Table 2).

3.3. Establishing standard curves for the species-/genus-specific, semi-quantitative amplification, and effect of different polymerases on the performance of the real-time PCR

In order to be able to estimate the relative proportion of species/genera contributing to a 'faecal egg count', standard curves were defined for the real-time PCR for individual species/genera of strongylid nematodes. The PCRs (using individual, specific primer pairs) were conducted on different days using serial titrations (0.1, 1, 10, 100 and 1000 pg) of genomic DNA from adult males of *H. contortus*, *Te. circumcincta*, *Tr. axei* and *Tr. colubriformis*, *Co. oncophora*, *Oe. columbianum* and *Oe. venulosum* or *Ch. ovina*, prepared on the same and on different days. Each of the seven standard curves determined for each assay (and each primer pair) was reproducible on different days, with mean *r* (correlation coefficient) values for individual curves ranging from 0.94 to 0.99 (see Table 3).

In order to increase the 'sensitivity' of the PCR (i.e. reduce the minimum amount of genomic DNA template detectable), the polymerase in the Sensimix dT kit (Quantace) was compared with GoTaq (Promega). A series of experiments was conducted using all seven primer pairs individually, testing reproducibility on different days. For some primer pairs and species (e.g., *Te. circumcincta*, *Trichostrongylus* spp. and *Oe. columbianum*), the minimum amount of genomic DNA required for effective amplification in the PCR was reproducibly smaller using the 'Sensimix' polymerase compared with GoTaq (see Table 3). Also, for each of the seven primer pairs,

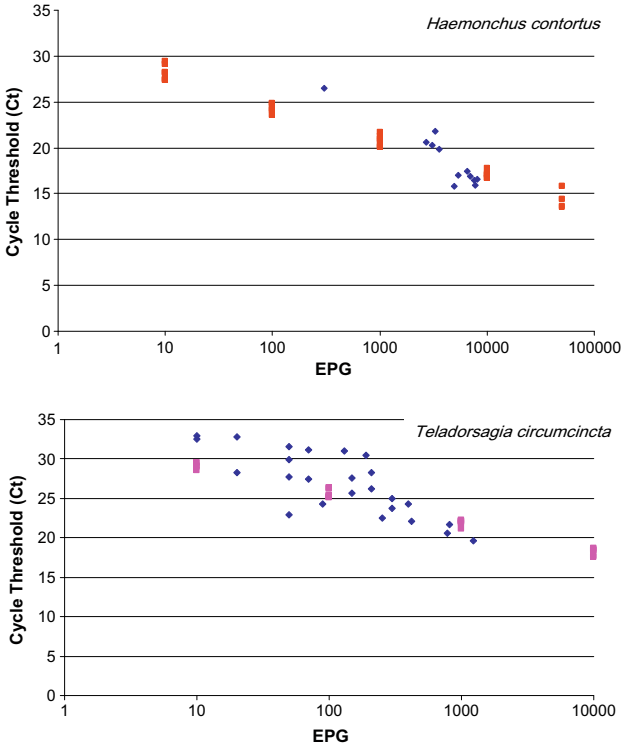

Fig. 4. Comparison of cycle threshold (Ct) values in real-time PCR with numbers of *Haemonchus contortus* or *Teladorsagia circumcincta* eggs per gram (EPG; $\log_{10}$-transformed) of faeces. PCR amplification was from genomic DNA purified from eggs from faeces from sheep with monospecific *H. contortus* or *Te. circumcincta* infection (closed diamonds), or directly from faecal samples spiked with defined numbers of eggs (closed squares) (see Section 2).

Table 3
Minimum amount of genomic DNA from which specific amplification of the ITS-2 region could be achieved in the PCR using individual primer pairs and each of two polymerases (GoTaq from Promega and SensiMix dT from Quantace), and the *r* (correlation coefficient) values for the standard curves obtained using the data for different amounts (0.1, 1.0, 10, 100 and 1000 pg) of genomic DNA for each nematode species representing each genus tested.

Species (primer pair)	Detection limit in pg		*r* value for standard curve	
	GoTaq	SensiMix	GoTaq	SensiMix
Haemonchus contortus (HAE–NC2)	0.1	0.1	0.89–0.98	0.95–0.99
Teladorsagia circumcincta (TEL–NC2)	2	1	0.91–0.98	0.95–0.99
Trichostrongylus spp. (TRI–NC2)	1	0.1	0.98–0.999	0.98–0.99
Cooperia oncophora (COO–NC2)	1	1	0.91–0.96	0.98
Chabertia ovina (CHO–NC2)	1	1	0.98–0.99	0.96–0.99
Oesophagostomum columbianum (OEC–NC2)	1	0.1	0.97–0.98	0.99
Oe. venulosum (OEV–NC2)	1	1	0.92–0.95	0.92–0.98

the kinetics of the reaction were such that the 'Sensimix' polymerase reproducibly achieved a greater amplification efficiency to reach the plateau phase than GoTaq. These results were largely reflected in a reduced cycle threshold (Ct) for each of the seven standard curves (Table 4) and a greater mean *r* value (see Table 3). The peak melting temperatures displayed for amplicons produced in duplicate on different days using each of the seven primer pairs and the 'Sensimix' polymerase, were usually ~2–5 °C lower than those generated using GoTaq polymerase (Table 5).

3.4. Specific amplification from genomic DNA purified from strongylid eggs from faecal samples from sheep with monospecific *H. contortus* or *Te. circumcincta* infection

Faecal samples ($n = 12$) from experimental sheep monospecifically infected with *H. contortus* were processed, EPGs determined and genomic DNA extracted from eggs. The EPGs ranged from 300 to 8030. Also, *H. contortus* eggs were added to faecal samples from helminth-naïve sheep: six samples were spiked with 50,000, 10,000, 1000, 100 and 10 EPG each. Two unspiked samples from helminth-free sheep were included as 'negative' controls. Genomic DNA was extracted from these samples using the C-Qentec™ service (see Section 2.3). The real-time PCR analyses of these samples (using primer pair HAE–NC2) revealed a correlation between EPG and Ct (Fig. 4), irrespective of method used for the isolation of genomic DNA. Specifically, for the samples with EPGs ranging from 300 to 8030, Ct values of 26.48 to 15.8 were recorded (Spearman's correlation coefficient [ρ] of $\log10_{EPG}$ and Ct: −0.804, $n = 12$, 95% confidence interval (CI): −0.943 to −0.428, $P < 0.0001$). For the samples with EPGs ranging from 10 to 50,000, the Ct values ranged from 29.42 to 13.50 (ρ of $\log10_{EPG}$ and Ct: −0.980, $n = 29$, 95% CI: −0.991 to −0.958, $P < 0.0001$).

Faecal samples ($n = 25$) from sheep known to be infected only with *Te. circumcincta* were also tested in the same manner as for *H. contortus*. The EPGs ranged from 10 to 1200. Also, *Te. circumcincta* eggs were added to faecal samples from helminth-naïve sheep: six samples were spiked with 10,000, 1000, 100 and 10 EPG each. Two unspiked samples from helminth-free sheep were included as 'negative' controls. Genomic DNA was extracted from individual samples using the C-Qentec™ service. The real-time PCR analyses of these samples revealed a correlation between EPG and Ct (Fig. 4), irrespective of method used for the isolation of genomic DNA. Specifically, for the samples with EPGs ranging from 10 to 1200, Ct values of 32.11 to 19.96 were recorded (ρ of $\log10_{EPG}$ and Ct: −0.8086, $n = 25$, 95% CI: −0.881 to −0.498, $P < 0.0001$). For the samples ($n = 12$) spiked with 10 to 10,000 EPG, the Ct values ranged from 29.52 to 17.63 (ρ of $\log10_{EPG}$ and Ct: −0.969, $n = 24$, 95% CI: −0.987 to −0.929, $P < 0.0001$).

No amplicons were produced from genomic DNA samples prepared from the faeces from helminth-free sheep. To ensure that a negative real-time PCR result (except the 'negative' controls) was not due to the inhibition of the PCR by faecal constituents, genomic DNA extracted from an adult of *H. contortus* was spiked into selected "helminth-free" samples and subjected to PCR amplification using primer pair NC1–NC2 (positive-control reaction). There was no evidence of PCR-inhibitory components in helminth-free samples. For both *H. contortus* and *Te. circumcincta*, the sequences determined from selected amplicons ($n = 20$–30 per species) were consistently shown to represent the appropriate species based on BLASTn analysis against reference sequences (GenBank Accession Nos. X78803 and X86026, respectively). Based on Ct values (see Fig. 4), the estimated intensity of infection of each of these two species could be categorised (see Table 6).

Table 4
Comparison of cycle threshold (Ct) values with [SD] using individual primer pairs and employing each of the two polymerases, GoTaq (Promega) and SensiMix dT (Quantace). A cycle threshold (Ct) of 0.05 was selected for comparison. $n = 25$–30 runs.

Amount of homologous DNA (pg)	Primer pair TRI–NC2		Primer pair OEC–NC2	
	GoTaq Ct	SensiMix dT Ct	GoTaq Ct	SensiMix dT Ct
1	29.1 [±0.83]	26.6 [±0.78]	30.4 [±1.4]	25.99 [±0.6]
10	24.5 [±0.34]	22.9 [±0.16]	26.17 [±1.36]	21.84 [±0.007]
100	18.9 [±0.22]	19.4 [±0.5]	21.74 [±0.72]	18.47 [±0.37]
1000	14.5 [±0.19]	13.4 [±1.1]	16.86 [±0.29]	13.84 [±0.02]

Table 5
Mean melting temperatures (°C) [SD], calculated based on data for separate analyses ($n = 6$–30) of PCR products amplified, using each of the two polymerases GoTaq (Promega) and SensiMix dT (Quantace), on different days.

Species	Primer pair	GoTaq	SensiMix dT
Haemonchus contortus	HAE–NC2	80.8 [±0.13]	78.05 [±0.15]
Teladorsagia circumcincta	TEL–NC2	81.0 [±0.20]	76.8 [±0.10]
Trichostrongylus colubriformis	TRI–NC2	80.3 [±0.06]	76.4 [±0.12]
Cooperia oncophora	COO–NC2	80.9 [±0.30]	78.2 [±0.06]
Chabertia ovina	CHO–NC2	85.4 [±0.12]	80.7 [±0.17]
Oesophagostomum columbianum	OEC–NC2	85.5 [±0.19]	82.4 [±0.17]
Oesophagostomum venulosum	OEV–NC2	82.5 [±0.17]	78.5 [±0.17]

3.5. Application of the real-time PCR/melting-curve approach to genomic DNA samples derived from strongylid eggs from faecal samples from sheep with naturally acquired infections

Having demonstrated the performance of the real-time PCR/melting-curve analysis for the specific, "semi-quantitative" diagnosis of monospecific strongylid infections (Sections 3.3 and 3.4), the approach was applied to eggs isolated from faecal samples from sheep ($n = 160$) with naturally acquired strongylid nematode infections, from five geographical regions in the state of Victoria,

Table 6
Results of the molecular testing of 160 faecal samples from individual sheep from different geographical regions in Victoria, Australia, using primer sets TRI–NC2 (*Trichostrongylus* spp.) and TEL–NC2 (*Teladorsagia circumcincta*). Estimated prevalence (%) and intensity of infection (Ct) for *Trichostrongylus* spp. and/or *Teladorsagia circumcincta* are given.

District (no. of faecal samples tested)	*Trichostrongylus* spp.	Range of intensities of infection, estimated based on Ct values	*Teladorsagia circumcincta*	Range of intensities of infection, estimated based on Ct values[a]	Mixed infections of *Trichostrongylus* spp. and *Teladorsagia circumcincta*
South-West Central (n = 43)	34/43 (79%)	16.78–30.98	41/43 (95%)	18.25–31.34	33/43 (77%)
Gippsland (n = 34)	33/34 (97%)	16.5–28.23	33/34 (97%)	16.54–31.67	32/34 (94%)
North-East Central (n = 27)	12/27 (44%)	22.82–28.28	15/27 (55%)	21.58–32.48	9/27 (33%)
North-East Highlands (n = 36)	23/36 (64%)	23.89–32.84	24/36 (67%)	23.77–32.03	18/36 (50%)
Western (n = 20)	11/20 (55%)	20.97–32.75	14/20 (70%)	16.02–26.33	10/20 (50%)
Total (n = 160)	113/160 (71%)		127/160 (79%)		102/160 (64%)

[a] Based on current data sets (see Fig. 4), intensities of infection can be estimated and categorised.

Australia. Individual samples were tested for the presence of *Trichostrongylus* spp. and *Te. circumcincta*, the two most common genera/species in Victoria (Besier and Love, 2003). The results are shown in Table 6. *Trichostrongylus* spp. were detected in 113 of 160 samples (71%); the lowest (44%; 12 of 27) and highest (97%; 33 of 34) percentages were recorded for the North-East Central and Gippsland areas, respectively. Sequences derived from selected amplicons (n = 25) represented *Tr. axei, Tr. colubriformis, Tr. rugatus* and/or *Tr. vitrinus*. *Te. circumcincta* was detected in 127 of the 160 samples (79%); the lowest (56%; 15 of 27) and highest (97%; 33 of 34) percentages were recorded in the North-East Central and Gippsland regions, respectively. Again, the sequences from selected amplicons (n = 25) represented *Te. circumcincta*. Mixed infection of *Trichostrongylus* spp. and *Te. circumcincta* were detected in 102 of the 160 samples (64%). The lowest (~33%) and highest (~94%) percentages of such mixed infections were in sheep in the North-East Central and Gippsland regions, respectively.

4. Discussion

In the present study, a real-time PCR platform was established for the specific and semi-quantitative amplification of DNA from *H. contortus*, *Te. circumcincta*, *Trichostrongylus* spp., *Co. oncophora*, *Oe. columbianum*, *Oe. venulosum* or *Ch. ovina* using, for each, a specific, forward oligonucleotide primer located in the ITS-2 and a conserved reverse primer (NC2) in the 5′-region of the large subunit of rDNA. Given that the eggs of *Nematodirus* can be readily distinguished from other economically important strongylids and that *N. battus*, the most pathogenic member of this genus (Bowman, 2009), has not been reported in Australia, the focus was on other key genera/species infecting sheep in this country. During the evaluation phase of the PCR, all amplicons (of appropriate sizes) generated using individual primer pairs, designed to the ITS-2 and 28S rDNA of the target species and genera, were sequenced to independently prove their specificity. Depending on the primer pair used, as little as 0.1–2 pg of DNA was sufficient to achieve specific amplification from the respective species, which equates to a proportion of the genomic DNA which can be isolated from a single egg (unpublished data). Using a panel of well-defined (heterologous) control DNA samples (see Table 1), there was no evidence of non-specific amplification, with the exception of a very faint amplicon from *Pa. ichikawai* DNA using primer pair OEC–NC2. However, both the size and melting-curve/peak of this amplicon were distinct from those representing each of the gastrointestinal strongylids included in this study. While the possibility cannot be excluded that non-specific amplicons might be produced from the genomic DNA of 'non-target' organisms in faecal samples from sheep, it is unlikely that the melting-curves of such non-specific amplicons would match those typical of the strongylid nematodes included in this study. Based on evidence from a range of previous studies, describing the use of ITS-1 or ITS-2 for the specific diagnosis of strongylid infections in hosts other than sheep (reviewed by Gasser, 2006; Gasser et al., 2008a,b), there is presently no evidence of problems with specificity in the PCR. Nonetheless, the specificity of the PCR using each of the seven primer pairs designed herein should be continually assessed in future epidemiological studies and in different geographical regions. Also, there was no evidence of inhibition in the PCR following the column-purification of genomic DNA from eggs, rendering the present approach more "sensitive" for the detection of eggs than the McMaster method (MAFF, 1977; whereby the detection of 1 egg equates to 30 eggs per gram of faeces).

The "standard curve" established for each of the seven test PCRs, using genomic DNA from individual species, revealed a linear relationship between Ct and log-transformed DNA amounts over four orders of magnitude. Similarly, such a relationship was demonstrated when the PCRs were assessed using DNA isolated directly using the commercial service C-Qentec™ (provided via SARDI, Adelaide, South Australia) from faecal samples spiked with 10, 100, 1000, 10,000 or 50,000 eggs of *H. contortus*, or 10, 100, 1000 or 10,000 eggs of *Te. circumcincta* per gram. Moreover, an assessment of the present real-time PCR platform using a panel of genomic DNA samples (n = 160) from nematode eggs derived from sheep (from six different regions in Victoria, Australia) with naturally acquired strongylid infections showed a significant correlation between Ct values and numbers of eggs per gram of faeces (for the nematodes detected). These findings show that the present PCR platform can be used for the semi-quantitation of DNA of target species/genera (*H. contortus*, *Te. circumcincta*, *Trichontrongylus* spp., *Co. oncophora*, *Oe. columbianum*, *Oe. venulosum* and *Ch. ovina*) in or directly from sheep faeces. Based on the present molecular data (i.e. Ct values), the intensity of *H. contortus* or *Te. circumcincta* for individual samples could be estimated (see Fig. 4). For example, for *Te. circumcincta*, predicted intensities were classified herein as: "L" (low; Ct of >29; $0 < EPG \leqslant 10$), "M" (medium; Ct of ~23–28; $10 < EPG < 300$) or "H" (high; Ct of ⩽22; $EPG \geqslant 300$). Such a categorisation (together with species/genus composition), rather than absolute quantitation, is considered appropriate, given that the intensity of infection (i.e. worms per sheep) does not necessarily reflect the number of eggs per gram excreted in faeces, with the exception of nematodes with high biotic potential, such as *H. contortus* and the Oesophagostominae. While not assessed herein, the effect of faecal consistency (cf. Le Jambre et al., 2007) on semi-quantitative PCR results might need consideration in future studies.

To allow the diagnosis of patent infections, the present study focused predominantly on the isolation of eggs immediately following sample collection. This approach was considered advantageous over the direct isolation/purification of DNA from faecal matter, because (i) eggs can be counted microscopically, thus yielding a "traditional egg count" and providing a reference for estimating proportions of eggs of particular species/genera based on the

semi-quantitative PCR results, (ii) eggs can be separated from the vast majority of faecal matter, thereby substantially reducing and almost eliminating faecal constituents (e.g., humic acids or polysaccharides) inhibitory to the PCRs, and because (iii) the disruption of eggs and subsequent isolation of genomic are considered much more efficient/effective compared with the direct extraction and purification of DNA from a large amount (i.e. 4 g) of faeces. The rapid isolation of genomic DNA from non-larvated eggs is critical, because mitosis during larval development (both within and outside of the egg) leads to a significant increase in cell number and thus copy number of ITS-2 in an individual, as supported by evidence from previous studies inferring enhanced amplification following incubation at room temperature for 6–24 h (Schnieder et al., 1999; Harmon et al., 2006). While not explored in the present study, faecal samples (of a defined amount [e.g., 4 g] and consistency) could be fixed in a volume of ethanol and cooled (4 °C) or frozen (at −20 °C or −70 °C) to overcome this potential "variable" and to enable processing (following sedimentation by centrifugation and washing in water) at a later stage. Some evidence (Verweij et al., 2007) indicates that the isolation of genomic DNA from eggs or worms from ethanol-fixed samples is efficient and allows transportation and storage at ambient temperature for some time. If required, such an approach of ethanol fixation and/or storage could be applied to faecal samples from sheep.

The diagnostic sensitivity and specificity (cf. Espy et al., 2006) of individual PCRs, based on the testing of eggs from faecal samples from a relatively large number of sheep with patent, monospecific infections with individual species, sheep with naturally acquired, mixed strongylid infections as well as helminth-free sheep, following autopsy and 'total worm counts', was not conducted for cost and logistical reasons. Such an extensive study could be carried out, but is unlikely to be essential or alter the findings of the present study, as there is no evidence of inhibition or non-specific amplification in the PCR (using each of the seven primer pairs), and the smallest amount of DNA from which amplification could be achieved was 0.1–2 pg, depending on species. These statements are supported by recent studies using similar approaches (Verweij et al., 2007; Nielsen et al., 2008). Nonetheless, infections in sheep by parasites, such as *Te. circumcincta* and *H. contortus*, which can undergo hypobiosis (arrested development) as larvae in the stomach wall (Gibbs, 1986), are unlikely to be detected, unless parasite DNA is released from the nodules in the gut wall into the ingesta or chyme to be specifically detected following PCR. In addition, while it is known that limited sequence polymorphism or heterogeneity in ITS-2 (usually <1%) may occur (among or within individuals from different geographical regions) within individual target species included in the present study (Gasser et al., 2008b), this variability is unlikely to affect the annealing characteristics of the primers used in the PCR or the amplification efficiencies, based on our experience. Nonetheless, the performance of the present PCR platform would need to be re-assessed whenever applied in another state of Australia or country.

The advantages of the present real-time PCR-coupled melting-curve analysis platform over conventional coprological and PCR approaches are that (i) the entire analysis is performed in a "closed-tube" format in a thermal cycler (Rotor-Gene 6000), eliminating the potential risk associated with 'cross-over' contamination; (ii) including genomic DNA isolation and purification, the entire procedure can be performed within 1–2 days to achieve a semi-quantitative result, with identification to the genus or species level, compared with 7–8 days for the technique of larval culture, (iii) the cost of the testing of one sample employing this PCR platform is estimated to be significantly less than other detection systems, such as *Taq*man probes (Heid et al., 1996), minor groove binder (MGB) Eclipse probes (Afonina et al., 2002), molecular beacons (Piatek et al., 1998) or fluorescence resonance energy transfer (FRET) (Chen and Kwok, 1999), although the latter systems are reported to ensure specificity in the PCR through exclusive binding to the target sequence (Monis et al., 2005b); and (iii) melting-curve analysis of individual amplicons independently confirms their specificity and identity following the PCR (cf. Monis et al., 2005a; Jeffery et al., 2007); this latter step essentially represents a mutation scanning analysis, which employs a dye (i.e. SYTO® 9; Monis et al., 2005a) that intercalates with double-stranded DNA. In this analysis, changes in fluorescence are recorded during an incremental heat-denaturation procedure, in which an amplicon gradually denatures into single-stranded DNA, leading to a decrease in fluorescence over time. The "melting peak" of a sequence or an amplicon displays the greatest change in fluorescence with increasing temperature (dF/dT) and is linked mainly to the nucleotide sequence, its composition (including G+C content) and the size of the amplicon. Hence, the melting-curve/peak of an amplicon essentially provides 'genetic' data, whereas amplicon size exclusively represents 'phenetic' information. It is important to ensure that the PCR conditions (including assessing different polymerases and concentrations of the buffers/salts and intercalating dyes) have been optimised, as changes in one or more of these parameters/components can affect the melting characteristics of a particular amplicon (see Monis et al., 2005a; Rasmussen et al., 2007; Reed et al., 2007). The principal advantage of melting-curve analysis over traditional mutation scanning approaches (such as single-strand conformation polymorphism [SSCP] or denaturing gradient gel electrophoresis [DGGE]) and direct sequencing is that no electrophoresis step is required, and denaturation takes place in minutes in solution within a closed-tube format. Typically, melting-curve analysis takes 15–20 min to carry out, following PCR, compared with ~6–16 h for SSCP or DGGE analyses (Gasser et al., 1998, 2006).

In conclusion, based on the findings presented and interpretations drawn, the present microscopic-molecular approach established could provide a useful tool for (i) routine diagnosis of strongylid infections in sheep in Australia and, with some adaptation, in other countries (depending on the range of nematodes present), (ii) epidemiological or ecology studies, e.g., for estimating the prevalence and distribution of key strongylids as well as the presence of drug resistance when combined with conventional "faecal egg count reduction testing", (iii) monitoring changes in parasite prevalence and distribution following treatment, and (iv) incorporation into targeted treatment and control programmes. Given the progress in defining specific genetic markers in nuclear rDNA (particularly ITS-2) of strongylid nematodes of livestock, in overcoming some of the limitations of inhibition in the PCR by components in faecal samples and in establishing selected PCR assays (also reviewed by Gasser et al., 2008a,b), the next goal of developing an advanced, high throughput (e.g., image-capture and robotic) system seems achievable. Given the prevalence of drug resistance in strongylid nematode populations of livestock, the definition of genetic markers linked to resistance (cf. von Samson-Himmelstjerna, 2006; von Samson-Himmelstjerna et al., 2007) is critical, such that they can also be included in any species- or genus-specific diagnostic assay.

While the focus of the present study was on strongylids of sheep, the findings and the approach taken should have implications for the development of assays for the diagnosis of strongylid nematode infections in other hosts, including goats, cattle, pigs, humans and non-human primates. Progress in this direction has been made by some workers (reviewed by Gasser et al., 2008a,b), but some assays require more extensive validation and enhancement before they can be employed routinely. While this study established a diagnostic platform to be used in a laboratory with real-time PCR facilities for routine testing, there is considerable interest in test systems that can be utilised "in the field" by the

farmer. Techniques, such as the loop-mediated isothermal amplification procedure (LAMP) (Notomi et al., 2000) and nucleic acid sequence based amplification (NASBA) (Compton, 1991; van Belkum and Neisters, 1995), show some promise for "field application", but require rigorous assessment. Irrespective of the format, a requirement for the development of any assay will be a reliable, universal technique for the rapid, direct isolation and subsequent enzymatic amplification of specific genetic loci from various types of biological matrices (i.e. faecal, pasture and tissue samples), depending on application (cf. Irvine and Dallas, 2002; Harmon et al., 2006); as there is no panacea for problems associated with the isolation of minute amounts of parasite DNA from different biological samples or for the removal of substances inhibitory to the enzymatic amplification process, methods might need to be optimised for each particular application.

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