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Rapid preparation of gastrointestinal nematode eggs from faeces for PCR identification

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ABSTRACT

Detection of gastrointestinal nematodes (GIN) as both a qualitative and quantitative test is highly desirable. Methods such as multiplex and qPCR are capable of providing such results, but can be laborious and expensive. This paper presents a rapid, low-cost method of preparing GIN egg from faecal samples that produces DNA suitable for PCR analysis. We also describe a set of primers that are suitable for single-tube multiplex PCR.

1. Introduction

Infection of livestock by gastrointestinal nematodes (GIN) results in considerable economic loss annually (Fanke et al., 2017). Symptoms of infection include malnutrition, weight loss, reduced product quality, anaemia and death (Roeber et al., 2013; Mohanraj et al., 2017). The detection and monitoring of GIN species is essential for timely and appropriate treatment of animals. Various detection methods are used to identify parasites and guide treatment. Quantitative tests such as faecal egg counts evaluate the severity of the infection, while qualitative tests such as larval cultures and enzyme-linked immunosorbent assay (ELISA) provide information on the species present. Polymerase chain reaction (PCR) has the capacity to be both qualitative (conventional PCR) and quantitative (quantitative PCR, or qPCR), by measuring the amount DNA amplified for specific species. Multiplex PCR and qPCR are particularly useful as they allow the simultaneous use of several primers sets, each targeting a species of interest. The proportions of infective species detected can be assessed and used to determine the most effective treatment. Selection of the appropriate primer sets to use for multiplex PCR tests requires careful assessment to ensure common annealing temperatures and extension times can be used, and that no secondary products with other materials or tertiary structures in any of the primer sets occur.

In addition to the selection of primers, attention to sample preparation is required, depending on the source of DNA, to prevent inhibition of PCR. Table 1 summarises the common PCR preparation techniques used to detect GIN. PCR can utilise DNA from adult nematodes extracted during necropsy, third stage larvae (L3) cultured from eggs in faecal samples, and DNA extracted directly from eggs. Collection by necropsy results in the most accurate quantification of species present but is generally not an option for maintaining livestock health under field conditions. Cultured larvae are easy to collect and provide a clean source of DNA for amplification. However, this requires additional time (several days) and introduces bias associated with culturing temperatures for specific species. Direct amplification of DNA from eggs is desirable as it reduces the time required for diagnosis and removes the bias inherent in culturing.

Extraction methods may be combined with kits, such as NucleoSpin Tissue kit (Macherey Nagel, Germany), DNeasy Blood & Tissue Kit® (Qiagen, Germany), Power Soil DNA Isolation Kits (MoBio, Germany) and Column purification with minicolumn (Wizard™ DNA Clean-Up, Promega, United States), to collect highly pure DNA.

Reliable PCR of DNA extracted from eggs requires removal of faecal debris and concentration of eggs. Conventional methods for cleaning and concentrating eggs involve filtration and flotation-based techniques. Concentrated eggs are then subjected to extraction techniques (Table 1), including bead-beating, commercial kits, freeze-thaw cycles, heat-treatment and sonication, to release and purify DNA. These techniques often involve several reagents, and multiple incubation and purification steps, which increases the time and labour requirements.

In this paper, we describe a rapid and simplified egg separation and DNA extraction protocol using a commercial electric milk cream separator (MCS). We have assessed the capacity of this protocol to generate high-quality DNA suitable for PCR analysis.

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Table 1
Common DNA extraction techniques for GIN.

DNA source	Extraction protocols	References
Adult worms	• Column purification • Ethanol DNA precipitation • Freeze-Thaw • Heat-treatment • Proteinase K incubation	Wimmer et al. (2004); Elmahalawy et al. (2018); McNally et al. (2013)
Eggs	• Ball milling, bead beating or vortexing with beads • Centrifuge/ Salt flotation • Column purification • Egg sedimentation • Freeze-Thaw • Heat treatment • Microwaving • Proteinase K incubation • Sonication • Sieving	Nielsen et al. (2008); McNally et al. (2013); Andersen et al. (2013a); Andersen et al. (2013b); Bott et al. (2009); Harmon et al. (2005); Santos et al. (2020); Amoah et al. (2020)
L3	• Sucrose centrifugation flotation • Bead beating • Egg sedimentation • Freeze-thaw	Mondragón-Ancelmo et al. (2019); Santiago-Figueroa et al. (2019); Sweeny et al. (2011); Learmount et al. (2009); Martínez-Valladares et al. (2012); Mohammedsalih et al. (2020)

Fig. 1. Motor Sich-100-19 centrifugal cream separator. Image source: http://www.motorsich.com/eng/products/consumers/separators/

2. Materials and methods

2.1. Collection of purified eggs using a milk cream separator

All samples for this study were provided from a veterinary clinic that diagnoses GIN infections. Collection of samples was done under supervision of a qualified veterinarian as part of routine animal care. All samples are from naturally infected sheep, information on the location, and ownership of the sheep was confidential and not shared. Faecal samples from ten naturally-infected sheep confirmed (by conventional flotation/microscopy) to contain all three target nematode species, *Haemonchus contortus*, *Ostertagia ostertagi* and *Trichostrongylus* spp., were pooled (minimum 3 g each) and homogenised in a small amount of saturated salt solution (Allfarm Animal Health, Hastings) to form a slurry. The slurry was agitated as it was passed through a 200 μm mesh sieve into an MCS (Motor Sich-100-19 centrifugal cream separator, Motor Sich, Zaporozhye, Ukraine) illustrated in Fig. 1, receiver bucket to a total volume of 1 L.

The MCS was switched on, allowed to reach maximum speed (30 s) and then the valve was slowly and gradually opened. Once the contents of the receiver bucket drained to the base of the valve, 500 mL of water was added in three equal portions. The water was poured around the rim of the receiver bucket and down the central stem to the valve to collect all the filtered material. This was followed by 500 mL of saturated zinc sulphate solution (Redox, Australia) added in three equal portions, then 1 L of water in three equal portions. Each portion of added liquid drained to the base of the valve before the next portion was introduced. Effluent from the 'cream' outlet was collected in a 25 μm sieve and back washed into a 50 mL centrifuge tube (ThermoFisher Scientific, Australia) with water. The collected material was centrifuged at 7000 $\times g$ for 5 min to sediment eggs. The supernatant was discarded and the tube refilled with deionised water. This was repeated until the supernatant was clear. The cleaned pellet containing eggs was resuspended in deionised water, distributed into 200 μL portions and stored at −20 °C.

2.2. DNA extraction methods

GIN eggs prepared by the MCS method (200 μL) were thawed on ice and DNA was extracted using the following methods:

2.2.1. Bead beating

A small amount of glass beads (Sigma, Australia) was added to 1.5 mL centrifuge tube before adding GIN samples and making the volume up to 400 μL with MilliQ water. Tubes were firmly capped and placed in a bead beater (MP Biomedicals, Australia) for 20 s at 4.0 M/S.

2.2.2. Sonication

Bleach was added to the GIN sample in a 1:10 (*v*/v) ratio. The sample was immediately capped and sonicated for 10 s, placed on ice, then neutralised using 1 M HCl (approximately 1:100 (v/v) ratio). Neutral pH was confirmed using litmus paper (Merck).

Table 2
Primer sequence details.

Name	Target	Sequence	Tm (°C)	Product length
O. ostertagi Forward	*O. ostertagi* isolate 232 internal transcribed spacer 1 (KX929994.1)	5′ TGG GAG TAT CAC CCC CGT TA 3′	59.66	73 bp
O. ostertagi Reverse		5' TCG CCA CTC ATG AAC GA CTC 3′	60.11	
H. contortus Forward	*H. contortus* isolate Sz9 internal transcribed spacer 2 (KC415119.1)	5' ACA TGT TGC CAC TAT TTG AGT GT 3′	58.86	72 bp
H. contortus Reverse		5' TCG TCG CCA TAC ATG TCA CT 3′	59.18	
Trichostrongylus Forward	*T. colubriformis* isolate VT161-C8_(Tco) internal transcribed spacer 2 (KU891930.1)	5′ TGT TCC TGT ATG ATG TGA ACG TG 3′	59.01	128 bp
Trichostrongylus Reverse		5' CGC CTG AGT TCA GGT TGC 3′	58.74	

2.2.3. Heat-treatment

GIN samples were held on ice, then placed in a 100 °C water bath for 5 min, followed by 5 min incubation on ice.

2.2.4. In-house "full" extraction method

GIN eggs were made up to 1 mL with MilliQ water and centrifuged for 1 min at 14,100 ×*g*. The supernatant was discarded, the pellet was resuspended in 500 μL extraction buffer (0.1 M Tris-HCl (Merck) pH 7.5, 0.05 M EDTA (Sigma) pH 8.0, 1.25% SDS (Sigma)) and transferred to a fresh microcentrifuge tube with glass beads. After adding 20 μL of lysozyme (Merk), the tube was thoroughly vortexed (Ratek), incubated on ice for 15 min, subjected to bead beating for 20 s at 4.0 M/S, then incubated at 80 °C for 5 min. The tube was cooled on ice for 5 min, then 250 μL cold 6 M ammonium acetate (Thermo Fisher) was added. Following thorough vortexing, the tube was allowed to stand on ice for 5 min, then centrifuged for 5 min at 14,100 ×*g*.

The use of bleach was also trialled with each of the bead beating and heat-treatment methods and neutralised as described above.

Following extraction, 600 μL of supernatant containing DNA was transferred to a new microcentrifuge tube containing 360 μL isopropanol (Merck). The contents were mixed by inverting the tube 5 times and DNA was allowed to precipitate for 5 min at room temperature. Following centrifugation for 5 min at 14,100 ×*g*, the supernatant was decanted and the fluid was drained on paper towel for 1 min. The DNA pellet was gently washed with 500 Ll of 70% ethanol (Thermo Fisher), then centrifuged for 5 min at 14,100 ×*g* and the supernatant was discarded. The tube was left open at room temperature for 10 min to dry the pellet. The pellet was resuspended in 100 μL MilliQ water and 2 μL RNase (10 mg/mL) (Sigma, Australia). The suspension was immediately tested using a Nanodrop 200 spectrophotometer (Thermo Scientific). Extracted DNA was stored at −20 °C.

2.3. PCR primer design

Primers (Life Technologies, Australia) were designed for three seasonally prevalent nematode species using the following GenBank accession numbers; *Haemonchus contortus* KC415119.1 (*Haemonchus contortus* isolate Sz9 internal transcribed spacer 2), *Ostertagia ostertagi* KX929994.1 (*Ostertagia ostertagi* isolate 232 internal transcribed spacer 1) and *Trichostrongylus* spp. KU891930.1 (*Trichostrongylus colubriformis* isolate VT161-C8_(Tco) internal transcribed spacer 2) (Roeber et al., 2017), as shown in Table 2. The three sets of primers were selected based on having similar melting temperatures, absence of secondary or tertiary structures, and no theoretical binding with each other with Multiple Primer Analyzer (Thermofisher) and OligoEvaluator™ (Sigma) online tools.

2.4. Polymerase chain reaction (PCR) amplification

PCR was performed using 15–70 ng of template, 0.5 μM of each primer and 17.5 μL Colourless Master Mix (Promega) in a final volume of 50 μL. Samples were heated at 95 °C for 1 min followed by 35 cycles of: 95 °C 15 s; 52 °C 30 s; 75 °C 30 s, then a 5 min soak at 75 °C and a final hold at 4 °C. PCR products were analysed in 2% agarose gels (Promega) electrophoresed at 40 V and 50 mA for 200 min in 1 x TBE solution (0.13 M Tris, 45 mM boric acid (Merck); 2.5 mM EDTA). The HyperLadder™ 25 bp (Bioline) was included in every gel.

2.5. Long term storage investigation

The DNA extractions prepared from each method were stored at −20 °C for 6 months. PCR was then performed on each sample to test if DNA was still able to be successfully amplified.

3. Results and discussion

Spectrophotometric analysis indicated that the greatest yield of nucleic acid was achieved with the methods utilising bleach with bead-beating, heat-treatment or sonication. However, no PCR products were

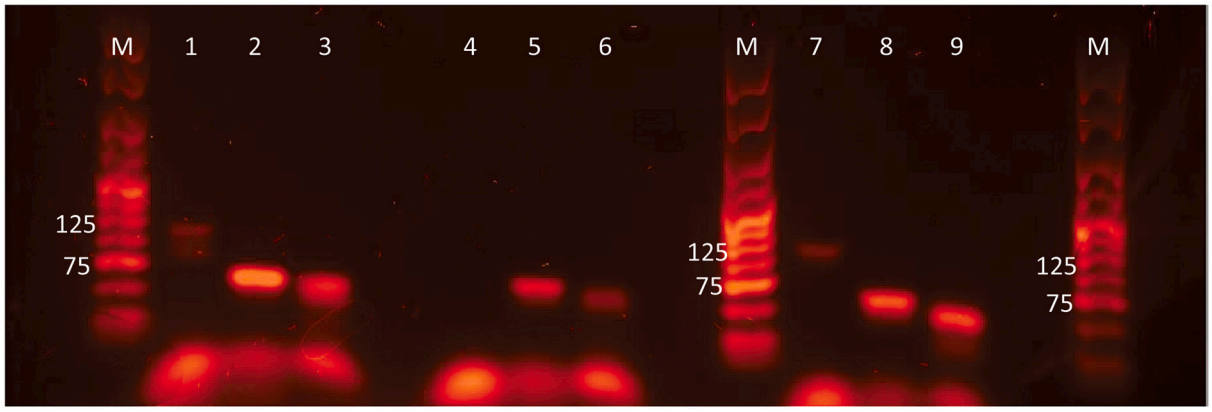

Fig. 2. PCR lanes from left to right: Heat treatment (1. Trichostrongylus spp., 2. *O. ostertagi,* 3. *H. contortus*), Bead beating (4. *Trichostrongylus* spp., 5. *O. ostertagi,* 6. *H. contortus*). DNA extraction (7. *Trichostrongylus* spp., 8. *O. ostertagi,* 9. *H. contortus*); M = Hyperladder markers.

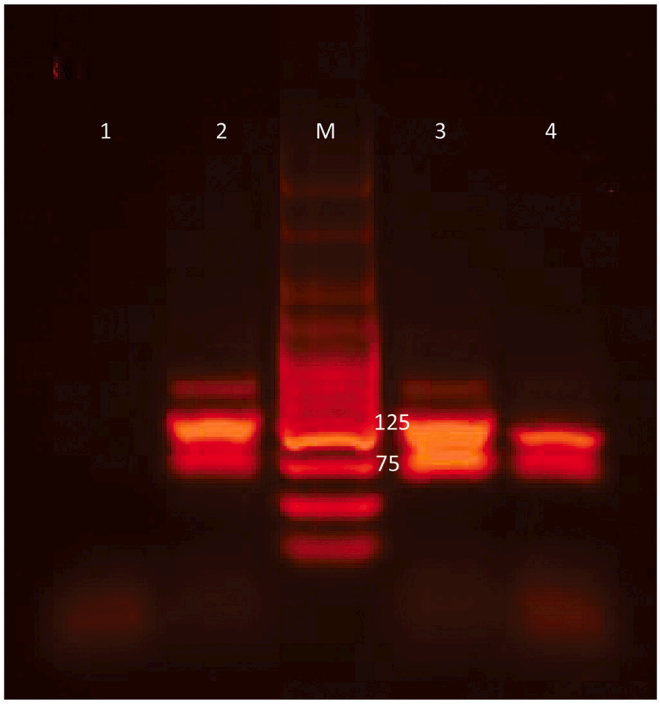

Fig. 3. Mixed primer PCR: 1. Sonication, 2. Bead-beating, 3. Full extraction 4. Heat-treatment; M = Hyperladder markers.

detected following electrophoresis in all samples prepared with bleach (supplement data). The abundance of nucleic acid but the absence of PCR product indicates that dilute bleach is highly effective at releasing DNA, but may have caused damage in the short timeframe of exposure prior to neutralisation. The use of bleach as an inexpensive and readily available substitute for ammonium acetate was found to be unsuitable for cell disruption, as the extracted DNA did not produce a band during gel electrophoresis following PCR. Ammonium acetate, which is commonly used in DNA extraction methods, was better suited and did not inhibit DNA amplification.

The processing of samples in MilliQ water without ammonium acetate also produced a DNA product that could be detected via PCR and gel electrophoresis. As such, while bleach is not a suitable substitute, ammonium acetate may be omitted from a method when not available or to reduce the number of reagents.

When all three primer sets were used simultaneously, gel electrophoresis showed a discrete band for *Trichostronglyus* spp. at approximately 120 bp. When each set of primers was used separately and compared via gel electrophoresis, the single base pair difference can be observed (Fig. 2). The bands for *O. ostertagi* and *H. contortus* were only 1 bp apart at 73 bp and 72 bp respectively. This resulted in a thicker blurred band that was difficult to distinguish (Fig. 3).

All three sets of custom primers were able to consistently amplify DNA products from bead-beating, heat treatment and "full" DNA extraction methods. If the proposed primers from these sets were to be used routinely, the addition of fluorescent tags, such as those used in qPCR, could distinguish the two amplicons of similar sizes. Alternatively, they could be used separately or one set could be altered to produce a different sized product for easier distinction via gel electrophoresis. Nonetheless, in our hands, the primer sets were performed well using DNA prepared with different methods.

The small portion volumes used here equated to 2 g of faeces, as used in a single animal test, showing that the method is viable for individual and bulk sample preparation. It is common to analyse 2-10 g of faecal matter for routine monitoring of GIN. A 10 g sample generally appears clear (minimal to no discolouration) when collected in approximately 10 mL of deionised water (unpublished), and the clean-up centrifugation between the MCS and pre-treatments steps is unnecessary. Instead, the sample only requires pelleting prior to pre-treatment.

The long-term storage of DNA had no visible impact on the quality of DNA prepared using the in-house DNA extraction method (Fig. 4). The bands for the heat treatment method appeared slightly fainter, but were still clearly observable. The DNA for the bead-beating preparation had become unable to be amplified after thawing, however, and the first PCR amplification produced very faint bands. A subsequent amplification of all three types found that the heat-treatment and in-house methods produced DNA that was still viable after thawing and storage at −20 °C for 6 months, while the bead-beating method produced higher volumes of DNA that lost its ability to be amplified after this extended storage

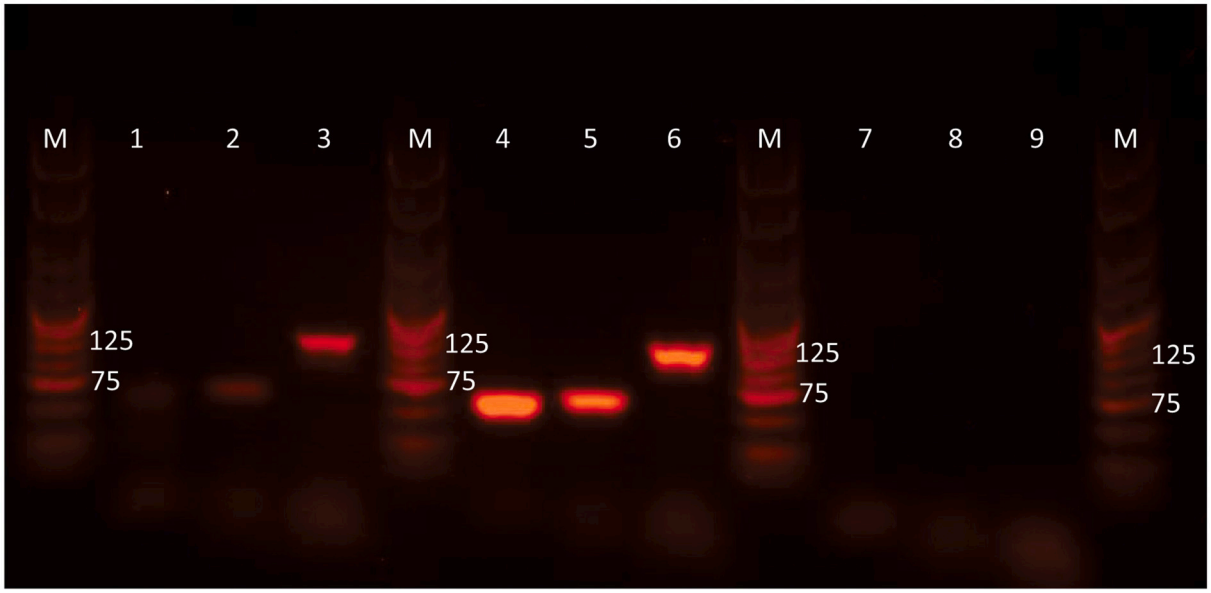

Fig. 4. PCR after long term DNA storage: Heat-treatment (1. *H. contortus,* 2. *O. ostertagi,* 3. *Trichostrongylus* spp.), Full extraction (4. *H. contortus,* 5. *O. ostertagi*, 6. *Trichostrongylus* spp.), Bead-beating (7. *H. contortus,* 8. *O. ostertagi,* 9. *Trichostrongylus* spp.)
M = Hyperladder markers.

period.

Hence, despite its excellent performance in the initial tests with fresh DNA, bead beating may be less viable for DNA that is to be stored prior to amplification and analysis. This, coupled with its simplicity, makes the heat-treatment method the most appealing rapid preparation option for PCR analysis of GIN eggs.

Overall, the most rapid and simple method that produced reliable results involved freezing of eggs prior to heat treatment in deionised water. The method required minimal reagents and equipment to produce DNA that resulted in identifiable bands after PCR and gel electrophoresis. Bead beating was also effective and had high DNA yields according to spectroscopy, but the difference was not observable after PCR amplification and required additional specific equipment. The MCS is effective at separating eggs from debris; it uses more solution than traditional flotation methods without compromising egg concentration. This results in a very clean and concentrated sample within minutes of processing. All necessary precautions should be taken to prevent cross contamination. It is worth noting that the internal discs in the MCS build up heavy (sinking) debris over time that contain a negligible number of eggs (unpublished) but require cleaning and reassembly. However, no instances of the sample blocking or inhibiting the flow of material were observed during processing.

4. Conclusion

Extraction of GIN eggs from faecal samples using the MCS was easy, rapid and produced a concentrated product for DNA extraction. The instrument provides a straightforward process for handling samples which can be implemented in any laboratory. The simplified DNA extraction method using heat treatment in deionised water provided reliable PCR screening results for the parasite species tested. The use of species-specific primers was reliable for identification even with amplicons differing in length by a single base pair. This preparation method and primers could be applied in qPCR with fluorescent tags for rapid, automated and quantifiable species identification.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106257.

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Glossary

Gastrointestinal nematode (GIN): Worm-like parasites that live in the gastrointestinal tract of their host.:
Multiplex: A variant of conventional PCR method that combines multiple reactions in a single test tube.:
Polymerase Chain Reaction (PCR): A process of replicating DNA or RNA for analysis.:
Stage 3 larvae (L3): Gastrointestinal nematodes in the third stage of their lifecycle, generally found after hatching and release from the faecal matter they were excreted in.: