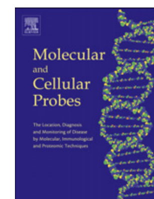
Molecular and Cellular Probes 27 (2013) 153–157

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr



Comparative evaluation of two DNA isolation techniques for PCR-based diagnosis of gastrointestinal nematode infections in sheep

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ARTICLE INFO

Article history:
Received 16 February 2013
Received in revised form
5 March 2013
Accepted 5 March 2013
Available online 21 March 2013

Keywords:
DNA isolation
Faecal samples
Sheep
PCR
Diagnosis
Strongylid nematodes

ABSTRACT

The specific diagnosis of gastrointestinal parasite infections in livestock is central to their control. PCR assays have been developed for routine diagnosis and to overcome limitations of classical methods. Central to the performance of such assays is the effective isolation of the nucleic acids from samples and the elimination of components that are inhibitory to PCR. Here, we directly compared two techniques for the isolation of DNA from strongylid nematode eggs from faecal samples from sheep, and assessed their performance in relation to the sensitivity and specificity of PCR, time required for DNA isolation and ease of use. The results showed differences in the performance of the two isolation techniques, subsequently effecting the PCR results. The main differences related to the time required for DNA isolation, and the elimination of inhibitory substances from the DNA isolated by one technique but not the other.

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The accurate diagnosis of gastrointestinal nematode infections of livestock underpins investigations of the biology, ecology and epidemiology of parasites and supports the monitoring of anthelmintic resistance [1,2]. To overcome the limitations of conventional methods of diagnosis of strongylid infections in sheep, we have established real-time PCR (RT-PCR) and multiplexed-tandem PCR (MT-PCR) assays that employ genetic markers in the internal transcribed spacers of nuclear ribosomal DNA (rDNA) [3–5]. These assays have high diagnostic sensitivity and specificity, and are able to reduce the time required for a specific diagnosis from one week (for conventional larval culture testing; cf. [5]) to one day. Given the semi-automated and relatively high throughput nature of the MT-PCR assay [3], the goal is to introduce this method into veterinary laboratories for routine diagnostic testing, requiring that the assay meet international standards [6,7]. A prerequisite to consistent, high assay performance is the effective extraction of DNA templates from test samples. Recent studies have shown that different DNA isolation kits can differ in their performance, particularly in relation to DNA yield and removal of components that are inhibitory to enzymatic amplification [8–10]. In spite of the excellent performance of the MT-PCR assay [3], we have observed, on occasions, partial inhibition of PCR for a small number of genomic DNA test samples, likely due to an ineffective removal of inhibitory components (e.g., polysaccharides, humic acids, phenolic compounds) from some samples upon DNA isolation and purification. Therefore, we elected to assess an alternative procedure for the isolation of genomic DNA from faecal samples from sheep to that employed originally [11]. Although there are numerous methods to choose from, including the QIAamp® DNA Stool Mini Kit (Qiagen Inc., Valencia, CA) or FastDNA® SPIN Kit (MP Biomedicals, Irvine, CA), we selected the PowerSoil® Fecal DNA Isolation Kit (MO BIO Laboratories Inc.), because results showed the suitability of this method for the isolation of PCR-amplifiable parasite DNA from the faeces from goats, cattle and deer [12–14]. However, this method had not yet been critically assessed for sheep faeces. Here, we directly compared the PowerSoil® kit with our previous DNA isolation method [11], and assessed MT-PCR performance with regard to diagnostic sensitivity (reflecting the removal of inhibitors), specificity, time required for isolation and ease of use.

Faecal samples ($n = 20$) were collected directly from the rectums of individual adult sheep following slaughter at a local abattoir. These sheep originated from a farm in Corowa, Victoria [36°00′S/146°23′E]. Individual faecal samples were placed into plastic bags, homogenized and weighed (15–20 g). Each sample was then divided into two equal size sub-samples (4 g), and faecal egg counts

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0890-8508/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.mcp.2013.03.002

(FEC) conducted [3] to enumerate strongylid nematode eggs. In brief, each sub-sample was homogenized in 60 ml of saturated sodium nitrate (specific gravity: 1.3), sieved (1 mm mesh size) and transferred to a 50 ml conical tube (Falcon); 1.5 ml of the suspension was aspirated using a sieve-top pipette, and three chambers of a McMaster chamber were each filled with 0.5 ml. Strongylid eggs were counted, and the mean number of eggs per gram (EPG) calculated. Subsequently, the remainder of each sub-sample was pre-prepared for DNA isolation. To do this, the 50 ml tube was centrifuged at 1000 *g* for 5 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; after centrifugation (2000 *g* for 5 min), the supernatant was removed and the pellet was washed again in the same manner; the final pellet of each sub-sample was re-suspended in 500 µl H_2O and transferred to a 1.5 ml Eppendorf tube and stored frozen (−20 °C). Genomic DNA was isolated from two sub-samples representing each individual faecal sample using isolation *methods A* and *B*, respectively. For *method A*, 250 µl of the first sub-sample were mixed with 100 µl of glass beads (1 mm in diameter, Sigma), 1% SDS and 500 µg/ml proteinase K (Boehringer) and incubated (in an orbital shaker) for 15 h at 37 °C. The suspension was centrifuged at 12,000 *g* for 10 min and the supernatant used for DNA isolation using a minicolumn (Wizard™ DNA Clean-Up, Promega); DNA was eluted in 50 µl of H_2O. For *method B*, 250 µl of the second sub-sample were used to isolate DNA using the PowerSoil® kit, precisely according to the manufacturer's instructions. Genomic DNA samples were stored at −20 °C until molecular analysis.

MT-PCR was performed as described previously [3], using the Easy-Plex system (AusDiagnostics Pty Ltd.), consisting of a Rotor-Gene 6000 real-time PCR thermocycler (Qiagen) and a Gene-Plex CAS1212 liquid handling robot (AusDiagnostics Pty Ltd.). Specific primers, designed to selectively amplify internal regions of the second internal transcribed spacer (ITS-2) sequences of *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus* spp., *Chabertia ovina* and *Oesophagostomum venulosum*, were used and are commercially available through AusDiagnostics Pty Ltd. (cat. no. 3809). Cycle threshold (Ct) values were recorded for each test-positive sample, and quantitative values for each parasite in each sample were determined using an automated comparison with Ct data determined for an internal spike-control (control-well containing 10,000 copies of a synthesized oligonucleotide template and a specific primer set) for each DNA sample tested [15]. DNA samples isolated using *methods A* and *B* were tested neat (=undiluted), and at 1/50 and 1/100 dilutions in MT-PCR; a 1/50 dilution was used for direct comparison of the two DNA isolation methods. All DNA samples were tested three times on different days. Samples in which the internal spike-control did not amplify or was significantly delayed from its expected cycle threshold value (i.e., >3) were considered as PCR-inhibited. Kappa statistics within the WinEpiscope 2.0 (http://www.clive.ed.ac.uk./winepiscope/) software package were employed to examine the agreement between the positive and negative test results obtained for samples isolated using the two different DNA isolation methods (at 1/50 dilution). Confidence intervals (CIs) were calculated using the exact binomial method in Stata v.12.0 (StataCorp, USA). PCR results were converted into percentages to estimate the contribution of particular nematodes to an observed faecal egg count (FEC) result; the quantitative results recorded in MT-PCR (given as number of DNA copies detected) for each of the five species or genera were added to calculate the total number of DNA copies detected per sample. The result recorded for an individual nematode was then divided by the total amount of DNA detected per sample and multiplied by 100 [3]. To examine the repeatability of the results obtained using undiluted DNA samples, the results of the three consecutive runs were examined for variation.

When tested at a 1/50 dilution (for direct comparison), the numbers of samples that were MT-PCR test-positive for particular nematode species/genus were similar for DNA isolation *methods A* and *B* (Table 1); 19 samples gave test-positive results (detecting one or more of the target nematodes) following both DNA isolation methods. For samples nos. 8, 14 and 17, microscopic results were test-negative. The DNA isolated from these samples by *methods A* and *B* gave PCR test-positive results for *T. circumcincta* (nos. 8, 14) and for *Trichostrongylus* spp. (no. 17) in repeat experiments (Table 1). There was no specific amplification for one sample, which was in accordance with the microscopic finding for this sample (i.e., no nematode eggs detected). With the exception of *Trichostrongylus*, the same or more DNA samples were recorded as PCR test-positive following *method B* compared with *method A* (Tables 1 and 2). The agreement in results between the DNA isolation methods, in conjunction with the MT-PCR, was 'substantial' to 'almost perfect' for all target nematodes, according to the definitions by Landis and Koch [16] (see Table 2). A discrepancy in results was observed for samples nos. 4, 7, 16 and 18 (Table 1). For sample no. 4, *Trichostrongylus* was only detected in DNA isolated using *method A*. For sample no. 7, *T. circumcincta* was detected only in DNA isolated using *method B*. For samples nos. 16 and 18, *H. contortus* or *T. circumcincta* were only detected in the DNA isolated using *method B*. For *H. contortus*, *T. circumcincta*, *Trichostrongylus* spp. and *O. venulosum*, 15%, 75%, 65%, 20% (*method A*) and 20%, 85%, 60% and 20% (*method B*) of samples were test-positive, respectively. For the majority of samples, the highest number of DNA copies detected in MT-PCR related to *T. circumcincta* and/or *Trichostrongylus* spp. (Tables 1 and 2). In only one sample did the highest number of DNA copies relate to *O. venulosum*. These findings are expected for this particular geographic locality and in accordance with our previous studies of these nematodes in the winter rainfall environment of Victoria, Australia [3–5].

When tested neat (undiluted), genomic DNA samples isolated by *method A* showed inhibition in PCR (Table 1). Partial or complete inhibition was reflected in retarded or no amplification of the internal spike-control in consecutive runs. A minimum dilution of 1/50 was required for DNAs isolated by *method A* to achieve specific amplification; this result was consistent for all samples tested. Although the 1/50 dilution was useful to achieve specific amplification from most samples, for sample no. 18, this dilution was not adequate. This sample showed a delayed amplification for the internal spike-control, and inhibition was still evident at a dilution of 1/100. When tested undiluted, genomic DNA samples isolated by *method B* achieved effective amplification of the target parasite species in consecutive runs; this finding was consistent for all samples. Dilution of these samples (1/50 and 1/100) neither improved the amplification efficiency in the MT-PCR nor did it change the numbers of parasites specifically detected in the samples.

The repeatability of the MT-PCR results was assessed on three consecutive days by testing DNA samples isolated using *methods A* and *B*. Although the results confirmed that there was no amplification using undiluted samples isolated by *method A*, undiluted samples isolated using *method B* showed high consistency when tested in consecutive runs. In 84% of observations, the difference between the results of three consecutive runs on the same samples was <5%. For 16% of observations, there was at least one of three comparisons for which the difference was >5% (in these samples the range was 6.54–50% of difference in the results). All samples were tested independently and blinded under the same conditions at the AusDiagnostics laboratory, Sydney, Australia. The results were entirely reproducible.

DNA samples isolated using *method B* was significantly more efficient at removing PCR-inhibitors than *method A*, as shown by

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