Copro-diagnostic molecular method for detection and identification of herpetofauna infecting nematodes

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Abstract

A non-invasive PCR-based methodology for sensitive detection and identification of parasitic nematode DNA released in the faeces of infected amphibians and reptiles as egg or tissue fragments (environmental DNA). A DNA extraction protocol optimised for liberation of DNA from resilient parasite eggs was developed alongside the design of a novel, nematode universal, degenerate primer pair that has broad-spectrum detection ability for shed nematode eDNA.

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Materials

QIAamp® Fast DNA Stool Mini Kit 51604 by Qiagen

FastStart™ Taq DNA Polymerase, 5 U/µl by Roche

Nem27 Primers by eurofins genomics

BSA 100X by New England Biolabs

GelGreenTM Nucleic Acid Gel Stain by Bioline

Hyperladder 1kb by Bioline

Agarose by Sigma

MiniElute® PCR Purification Kit by Qiagen

Protocol

Step 1.

DNA was extracted from a starting faecal quantity of 10-200 mg (depending on obtainable amount) using the QIAamp® Fast DNA Stool Mini Kit (Qiagen) under aseptic conditions using the manufacturer's protocol alongside the following modifications.

Step 2.

A disruption step was included in which the faecal samples were added to 1 ml of InhibitEx buffer followed by bead-beating using 4 mm diameter borosilicate glass beads (Sigma) placed within an Eppendorf Safelock 2 ml test tube. Samples were then bead-beaten in a Retsch MM400 mixer mill (Derbyshire, UK) at 30 Hz for between 5 – 10 min with regular movement of the samples between the pockets of the arm cradles to ensure a consistent beating across all samples.

Step 3.

Samples were vortexed for one minute and then incubated and shaken in an Eppendorf Thermomixer C (Stevenage, UK) at 45^{0}C and 67 g for between 1 – 2 hours.

Step 4.
Proteinase K digestion was carried out for 20 min.

Step 5.
Two elution steps were typically carried out, a first elution for 20 min in 100 µl of buffer AE with centrifugation, followed by a second elution step in 50 µl for 15 min and centrifugation.

Step 6.
When not in use DNA samples were kept chilled at 4^{0}C.

Step 7.
PCRs were prepared in aseptic conditions with all consumables UV sterilised, mastermixes were made on ice. PCRs were typically 25 µl in volume comprising: 10.88 µl of Mili-Q water, 2.5 mM PCR buffer, 3.5 mM Mg, 0.5 µM dNTPs, 0.024 U/µl FastStart Taq DNA Polymerase (Roche, Sussex, UK), 0.5 µM of both forward and reverse primers and 0.5 µl BSA (100X) (New England Biolabs Inc., Hitchin UK).

Step 8.
5 and 10 µl of faecal DNA was used per reaction. Tissue DNA extracts typical contained 10 – 50 ng/µl and faecal extract from 5 – 100 ng/µl.

Step 9.
Degenerate nematode specific primers developed in this study (Nem27 primers) comprised Nem1217F which had the 3'-5' sequence CGN BCC GRA CAC YGT RAG and Nem1619 which had the 3'-5' sequence GGA AAY AAT TDC AAT TCC CKR TCC. Nem27 primers amplify a 402 bp fragment of the 18S rRNA gene.10. DNA amplification was carried out using an initial denaturation at 94^{0}C for 5 min; 35 cycles of amplification (94^{0}C for 30 s; 54^{0}C for 30 s; 72^{0}C for 1 min); followed by a final extension at 72^{0}C for 10 min. Note: Nem27 primers could amplify nematode DNA from a faecal background at annealing temperatures as high as 62^{0}C to 64^{0}C, reducing the likelihood of non-specific amplification.

Step 10.
PCR products were run and visualised on 1% agarose gels comprising molecular grade agarose (Bioline, London, UK), TBE buffer and 0.5 – 2 µl GelGreenTM Nucleic Acid Gel Stain (Biotium, Cambridge, UK). To load gel, 3 µl of PCR product was added to 2 µl of blue loading buffer (Bioline) and pipetted into the wells alongside 1 µl Hyperladder 1kb (Bioline) size standard. Product sizes were separated using electrophoresis in a RunOneTM Electrophoresis Cell (Cheshire, UK) at 45 v for between 30 – 80 min, depending on the size of the gel.

Step 11.
Gels were mounted on a PrepOneTM Sapphire illuminator (EmbiTec) covered by a PI-1002 PrepOneTM filter (EmbiTec) and camera hood and photographed.

Step 12.
PCR product amplicons were cleaned using a MiniElute® PCR Purification Kit (Qiagen), with slight modifications to the manufacturer's protocol. Cleaned DNA was eluted in 10 µl of autoclaved Milli-Q water for 20 minutes.

Step 13.
10 – 40 ng/µl of cleaned PCR product was added to 4 pmoles of a single relevant primer and the final volume adjusted to 10 µl using Milli-Q water. Samples were Sanger sequenced at the University of Manchester DNA Sequencing Facility using Big Dye 3.1 chemistry on an ABI 3100 Genetic Analyzer (Fisher Scientific).

Step 14.

Sequence traces were examined and regions of poor quality or low-confidence sequence were removed in BioEdit. The complimentary sequence of that produced by the reverse primer was aligned next to the sequence produced by the forward primer, using the ClustalW function. This allowed for the extraction of the entire DNA sequence amplified by the primers.

Step 15.
To identify the species from which the sequences were from they were run through the GenBank nucleotide BLAST tool.