National Reference Laboratory

Standard Operating Procedure: DOC36

Identification of anisakid larvae at the species level by sequencing analysis

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Introduction

Several species of nematodes from the family Anisakidae and occasionally from the family Raphidascarididae are the etiological agents of the zoonotic human disease known as anisakiasis. It includes species from different genus such as Anisakis (*e.g. A. simplex, A. pegreffi, A. ziphidarium, A. physeteris and A. typica*), Pseudoterranova (*P. decipiens, P. krabbrei and P. bulbosa*), Contracaecum (*e.g. C. osculatum*, *C. rudolphii*) and Hysterotilacium (e.g. *H. aduncum*). The consumption of raw or undercooked fish or cephalopods infested with anisakid larvae is the main route of transmission of the disease to humans and other mammals. Identification of anisakid larvae and adults at the species level based solely on morphological criteria is often not reliable. Several molecular biology methods have been developed for anisakids identification. These methods are often based on indels or nucleotide sequence variation among anisakids species.

Scope

This standard operating procedure (SOP) describes the extraction of DNA from individual larva from the family Anisakidae, amplification of the complete Internal Transcribed Spacer (ITS, including ITS-1, 5.8S, ITS-2) by PCR and purification of amplicons for Sanger sequencing. The analysis of nucleotide sequences obtained is also described in this SOP.

Equipment and materials

Nitrile gloves

Laboratory coat

Variable volume pipettors p10, p100, p200, p1000 and respective filter tips (e.g. Rainin pipet lite XLS)

Plastic autoclaved racks

Mini centrifuge (e.g. Stuart Spinner SCF1 Bibby Scientific)

Vortexer (e.g.Stuart SA8 Bibby Scientific)

Waste bag

0.5 ml PCR tubes

1.5 ml microfuge tubes

1.5 ml Eppendorf SafeLock tubes

2 ml lysing matrix A tube

Extraction cabinet (e.g. Labconco purifier PCR enclosure)

Fridge in room 213 (Moff7)

Freezer (Small) room 213

Centrifuge (e.g. Heraeus Biofuge Pico)

Thermal cycler (e.g. DNA Engine Tetrad 2, Peltier Thermal Cycler)

MP Biomedicals™ FastPrep -24™ Classic Instrument

Heat Block at set to 55°C and 70°C

Nanodrop 2000 Spectrophotometer

Eurofins Genomics TubeSeq Labels

Chemicals

70% Ethanol in a spray bottle

1% Virkon (1 tablet in 500mls H_2O) in a spray bottle.

GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma)

100% Ethanol

GoTaq® flexi DNA polymerase PCR mix (Promega)

Deoxynucleotidetriphosphates (dNTPs) mix (Promega)

Primers 10 µM (see Table 2)

Molecular biology grade water

Positive control DNA *A. pegreffi*

Wizard® SV Gel and PCR Clean-Up System (Promega)

Method

A laboratory coat and gloves must be worn for all stages.

DNA Extraction Using GenElute™ Mammalian Genomic DNA Miniprep Kits, Sigma

In room 213, clean surfaces, pipettes and vortexer using 1% Virkon solution followed by 70% ETOH with paper towel.

Transfer ethanol preserved anisakids larvae into a petri dish using a wide bore pipette tip transfer individual larva to a 2 ml lysing matrix A tube (MP Biomedicals) containing 180 µl of lysis solution T (GenElute™ Mammalian Genomic DNA Miniprep Kits, Sigma). Homogenise at 5 m/s for 40 seconds using MP Biomedicals™ FastPrep -24™ Classic Instrument (MP Biomedicals). Add 20 µl of proteinase K solution (20 mg/ml), briefly vortex and incubate at 55 °C for 3 hours on a heat block. During the incubation, vortex occasionally. At the end of the incubation, vortex briefly, spin and add 200 µl lysis solution C (GenElute™ Mammalian Genomic DNA Miniprep Kits, Sigma). Vortex thoroughly during 15 seconds and incubate at 70 °C for 10 minutes on a heat block. Prepare the column by adding 500 µl of column preparation solution (GenElute™ Mammalian Genomic DNA Miniprep Kits, Sigma) to each pre-assembled GenElute™ Miniprep binding column and centrifuge at 10,000 x g for 1 minute. Discard flow-through liquid. Add 200 µl of 100% ethanol to the lysate and vortex thoroughly for 5-10 seconds to mix. Transfer the entire mixture to a treated binding column and centrifuged at 10,000 x g for 1 minute. Discard the collection tube and transfer the binding column to a new collection tube. Ensure that ethanol has been added to the concentrated wash solution by looking for a tick on the top of the bottle, if no tick is present add the volume of ethanol denoted on the side of the bottle. Add 500 µl of wash solution (GenElute™ Mammalian Genomic DNA Miniprep Kits, Sigma) to the binding column and centrifuged at 10,000 x g for 1 minute. Discard the collection tube containing the flow-through liquid and insert the column into a new collection tube. Add 500 µl of wash solution to the column and centrifuge at 16,000 x g for 3 minutes. Discard the collection tube with the flow-through liquid and insert the column into a new collection tube. Centrifuge at 16,000 x g for 1 minute with the lid off to remove residual ethanol. Transfer the column to a new collection tube and pipette 50 µl of elution solution into the centre of the binding column (GenElute™ Mammalian Genomic DNA Miniprep Kits, Sigma).Incubate at room temperature for 5 minutes. Centrifuge at 10,000 x g for 1 minute to recover the eluate to a new tube.

Amplification of the ITS rRNA by Polymerase Chain Reaction (PCR)

Prepare the PCR master mix using the volumes shown in Table 1for each sample to be tested plus 4 additional reactions (one for the negative control, one for the positive control and two spare). Vortex the master mix, spin down and pipette 47.5 µl into a 0.5 or 0.2 ml PCR tube for each sample. A negative control and a positive control must be run for each PCR.

Table 1: PCR Master Mix components

Reagent	Volume (µl)
5X Flexi Buffer (Promega)	10
$MgCl_2$ (25mM) (Promega)	3
dNTPs (25mM) (Promega)	0.4
NC5 primer (10µM)	1
NC2 primer (10µM)	1
Molecular Grade Water	31.85
Taq polymerase (Promega)	0.25
Total	47.5

Return any excess components of the PCR master mix back into the -20 °C freezer in 213, to limit the potential for contamination of stock tubes and remove the extracted DNA from either the fridge (if extracted immediately prior to this PCR) or the freezer. Mix DNA by pipetting up and down and add 2.5 µl to the PCR master mix. For the negative control use 2.5 µl molecular biology grade water and for the positive control 2.5 µl of *A. pegreffi* DNA (to be added to the tube in room 233).

Replace caps on all tubes, vortex and flash spin to mix contents and to ensure that the PCR mix is at the bottom of the tube. Remove the rack containing the PCR tubes and clean the cabinet with 1% Virkon, followed by 70% ethanol and UV for 5 minutes.

In room 233 place the tubes into the thermocycler and run a program with the following parameters according to the EULP protocol: Denaturation at 95 °C for 2 minutes followed by 35 cycles of 95 °C for 30 seconds, 55°C for 30 seconds and 72 °C for 75 seconds followed by a final elongation at 72 °C for 7 minutes and a 10 °C hold.

The PCR products can be stored either in a labelled bag in the fridge in 213 or for long term storage the freezer in room 213.

Table 2: Primers used for amplification of ITS of Anisakid nematodes

Name	Primer sequence (5'-3')	Forward/Reverse	Reference
N5	GTAGGTGAACCTGCGGAAGGATCATT	Forward	Zhu et al. (1998)
N2	TTAGTTTCTTTTCCTCCGCT	Reverse	Zhu et al. (1998)

Agarose Gel Electrophoresis

Run 10 µl of each PCR product on a 2% agarose gel according to the guidelines in SOP 1742. The size of the PCR products obtained should range between 898 and 953 bp depending on the species. The size of amplicons expected for *A. pegreffi* is 951 bp..

Purification of PCR Products

Add an equal volume of Membrane Binding Solution (Wizard® SV Gel and PCR Clean-Up System, Promega)to the PCR products. Insert SV minicolumn into a collection tube and transfer the prepared PCR product to the minicolumn assembly. Incubate at room temperature for 1 minute and centrifuge at 16,000 x *g* for 1 minute. Discard the flowthrough and re-insert Minicolumn into a collection tube.. Add 700 µl of wash solution (Wizard® SV Gel and PCR Clean-Up System, Promega) centrifuge at 16,000 x *g* for 1 minute. Discard the flowthrough and re-insert Minicolumn into a collection tube. Add 500 µl of wash solution and centrifuge at 16,000 x *g* for 5 minutes. Discard the flow-through and re-insert Minicolumn into a collection tube. Centrifuge the minicolumn at 16,000 x *g* for 1 minute to remove residual ethanol. Transfer the minicolumn to a clean 1.5 ml microcentrifuge tube and add 50 µl of nuclease free water (Promega).Incubate at room temperature for 1 minute. Centrifuge at 16,000 x *g* for 1 minute to recover the eluate.

Preparation of amplicons for Sanger sequencing

Quantify the purified PCR products by analysing 2 µl using a Nanodrop 2000 Spectrophotometer. Dilute PCR product to 5 ng/µl in a total volume of 15 µl in molecular grade water in an 1.5 ml Eppendorf SafeLock tube. Add 2 µl of 10 µM NC5 primer (Table 2) to tube. Eurofins Genomics TubeSeq label stuck to tube and sent in a padded envelope to: Sequencing Collection Wolverhampton, Eurofins Genomics, i54 Business Park, Valiant Way, Wolverhampton, WV9 5GB, United Kingdom. Analyse the nucleotide sequences obtained by BLASTn analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Review

Yearly

References

Identification of Anisakidae Larvae at the species level by PCR/RFLP. European Union reference laboratory for Parasites. Department of infectious, Parasitic and Immunomediated diseases. Unit of gastroenteric and tissue parasitic diseases. Istituto Superiore di Sanita.

TubeSeq Service Sample Submission & Ordering Guide. Eurofins Scientific. Luxembourg.

Zhu, X., Gasser, R.B., Podolska, M., Chilton, N.B., 1998. Characterisation of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. Int. J. Parasitol. 28, 1911-374