PT-07: "Molecular identification of Anisakid nematodes at the species level"

Instructions

PT ITEMS

Description. Each PT item consists of a 1.5ml tube containing a single fragment or DNA of Anisakidae L3 larvae. The PT panel consists of four tubes: two containing a single fragment of Anisakidae L3 larva of different species and two containing DNA extracted from a single Anisakidae L3 larva belonging to different species.

Production. L3 larvae are isolated from naturally highly infected fishes and stored in 96% ethanol. DNA is extracted from a single or a fragment of L3 larva.

Distribution. Each larva is cut into several fragments; any single fragment is individually transferred into a tube and filled with 96% ethanol. For DNAs, an aliquot (10µl) of DNA extracted from a single L3 larva is transferred into a tube. Due to the nature of the PT items, production and distribution are made some days before the shipping data and PT items are stored at a temperature below +15°C until shipping.

Labeling. Each PT item is plugged and sealed with plastic film, labeled with a unique code not attributable to larvae species.

Homogeneity check. All larvae and DNAs have been individually identified at species level by analyzing one of their fragments by the EURL-P internal method, accredited according to ISO 17025 "Identification at species level of parasites of the family Anisakidae by PCR/RFLP". The DNAs extracted from single larvae were also identified at species level by the method mentioned above. Homogeneity is further ensured by providing all participants with an aliquot of the same DNA preparations.

Packaging. Each PT panel (consisting in four PT items) are then placed in a larger container (e.g. 50ml tube) to ensure that they remain grouped and protected during handling and transport. The larger container is labelled with participant code. The PT panel is placed inside a polystyrene carton, ready for shipment. Several ice packs are arranged in the package to maintain internal temperature below +15°C.

Stability and quality control. The stability of the PT items has been evaluated by *ad hoc* experiments carried out by EURL-P. Larvae and DNAs preserved in 96% ethanol and stored at a temperature below +15°C maintain their stability for up to five years. Quality control requires that PT provider's staff analyses PT items before distribution. A fragment of each larva and each DNA are tested to confirm their identity. Species identification is performed by EURL-P internal method, accredited according to ISO 17025 "Identification at species level of parasites of the family Anisakidae by PCR/RFLP".

SUBMIT A PARTICIPATION REQUEST

The PT provider (EURL-P) announces the PT via e-mail addressed to potential participants. The email provides information on: PT items shipping date, PT scheme scheduled, the link to the participation form and the deadline, a summary of all deadline dates for the different phases of the PT scheme and the link to the PT provider's website for further information on PT scheme.

Participation request has to be submitted by completing the participation form (PT request form) within the due date indicated in the form and in the email.

At the time of PT items shipment, the PT provider sends participants the link to the on-line form where participant is requested to provide the following information:

- Package content and its condition of preservation.
- Timing of analysis
- Materials and Methods used to analyze PT items.
- Results.

PT ITEMS STORAGE

- Store PT items refrigerated at a temperature below +15°C until the analysis is performed.
- Check the packaging and its contents for correctness and completeness: four PT items properly labeled and sealed.
- Record the verification of the packaging upon arrival in the on-line form (see point 3).
- In case of discrepancy, fill in the claim form available at EURL-P website: https://www.iss.it/documents/20126/6678302/Claim-Appeal-rev-1.pdf/f9bba72e-3032-88fd-9d05-2b04ad45d1f1?t=1716802008339 or contact via e-mail the person in charge of the PT.

PT ITEMS ANALYSIS

1. The code assigned to participant is reported both on a paper sheet contained in the package and on the secondary envelope containing the PT panel.
2. PT items treatment:
   - Treat PT items as routine samples
   - Record the codes reported on the larger container (participant code) and on each PT item (PT item identification code)
   - Whenever transferring larvae from the original tube, use a small volume of ethanol (2-5µl). Wait for ethanol to evaporate completely before starting the DNA extraction
   - Commercial kits based on magnetic beads, which are generally more efficient than other systems, are recommended for DNA extraction from single larvae or fragments. Alternative DNA purification methods (e.g., silica membrane-based kits) can be also used. In the latter case, reduce the elution volume to 50–80 µL (if permitted by the manufacturer) to increase DNA concentration
   - The use of high quality Taq polymerase (e.g. hot start) reduces the risk of obtaining non-specific PCR products
   - Whenever multiplex PCR is applied, the quality of oligonucleotide synthesis may affect the efficiency of PCR reaction
3. Analysis: each participant is requested to apply any molecular method suitable for the purpose of the scheme, preferably the one used routinely.
4. Results: each participant is requested to indicate the assigned code, the PT item codes and the Anisakidae larvae species of each PT item. It is not allowed to submit results after the due date.

   Results have to be reported in the on-line form addressed to participants by PT provider on the same day the PT items are shipped.

   In the on-line form, participant has to indicate full reference if a published method is applied and any relevant deviation from the chosen method.
5. Deadlines:
   - Shipping is usually performed on Monday
   - PT items should be delivered within 48-72 hours
   - The deadline for results submission is indicated in the annual timetable available on the EURL-P website (https://www.iss.it/en/eurlp-prove-valutative-interlaboratorio), in the PT request form and in results form.
   - The final PT report is published on the EURL-P website according to the annual timetable available online (https://www.iss.it/en/eurlp-prove-valutative-interlaboratorio)
6. Perform evaluation: Result evaluation is qualitative. Result is considered “correct” if larvae and DNAs of the PT item are properly identified at species level, and “incorrect” in case of wrong identification. Results have to be expressed reporting the species assigned to each PT item. PT provider evaluates the result by the participant according to the sensitivity of the method applied. The participant is asked to correctly identify at least the species, for hybrid genomes (i.e. *A. simplex/A. pegreffi* hybrid genotype) the result will be evaluated based on the genetic marker used (i.e. mitochondrial vs nuclear markers). Likewise, the correct identification of the subspecies will be evaluated according to the resolution of the method applied. Due to the nature of PT items, no statistical parameters are applied for result evaluation. Each result is evaluated against the value assigned by the PT provider. The final evaluation is “positive” if all PT items are correctly identified, otherwise it is “negative”.
7. Report: PT provider drafts the Final PT Report, including, for each participant, information on:) the expected species per PT item; ii) the species identified by the participant per PT item; iii) the final evaluation and iv) recommendation based on participant performance and an updated summary of participant performance over successive PT rounds. To ensure confidentiality each participant is identified by an alphanumeric code changed annually. The final PT report is published on the EURL-P website (https://www.iss.it/en/rapporti-finali-prove-valutative-interlaboratorio) and presented to the National Reference Laboratories during the annual workshop.
8. Follow up: in case of negative result, the PT provider contacts the participant who fails the PT and sends a link to a form where the participant is asked to analyze the causes of failure and indicate the corrective actions planned or implemented.

9. Confidentiality: EURL-P retains the PT Reports for 10 years. PT provider staff signs a confidential agreement regarding acquired data. Individual PT report is available only to the participant in the restricted area of the website. In the final PT report, each participant is identified by alphanumeric code. The PT provider reserves the right to provide participant PT results to competent authority and accreditation body, upon request. In such cases, the PT provider informs the participant in writing.

For any information or problem. related to the web site access, please address:

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