Reissue n° 1 Final report PT-07: AnMol 1/2025

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

Reason for reissue:
The report was reissue due to the presence of mistaken extra sentence in the Comments section

This document cancels and replaces Final report PT-07 AnMol 1/2025

Design

Purpose	Evaluation of laboratories competence in molecular identification of Anisakid nematodes species	
Timetable	Invitation e-mail: within 07/02/2025 Website updates: within 07/02/2025 Registration deadline: 24/02/2025 PT items production: within 17/03/2025 Shipping: 17/03/2025 Results submission deadline: 11/04/2025 Publication of the Final PT Report: within 31/05/2025	
Participants	National Reference Laboratories for Parasites, Public and private, national and international institution	
Number of participants	Depending on requests	
PT items	Matrix	Ethanol (larvae) and saline buffer (DNA)
	Item	Anisakid nematodes (DNAs or larva fragments)
	Number of PT items	Based on request
	Panel composition	4 per each participant (2 larva fragments and 2 DNA items)
	Number of surplus items	n.a.
Activities provided by external providers	Shipping	
Provider name	DHL	
Results evaluation	Qualitative evaluation	

Implementation

PT staff: *Francesco Celani, Antonio Di Grazia, Irene Tartarelli*

Compliance with planned timelines: YES ■ NO ☐

Participant number and type: 17 public institution

0 private institution

Acquisition of matrix and analyte: L3 larvae stored in 96% ethanol were obtained by qualified providers or by EURL-P. DNAs were extracted from a single or a fragment of L3 larva. Fragments of larvae and DNAs were characterized at species level by accredited EURL-P internal molecular method (MI-04: Identification of Anisakidae Larvae at the species level by PCR/RFLP) on 04/03/2025.

Production of PT items: The PT items were produced on 11/03/2025 and consists of single fragment (in ethanol) or 10 µl of Anisakidae L3 larvae DNA. The PT panel consisted of four 1.5 ml 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.

Activities provided by external providers: The PT provider (PTP) entrusted the shipment of PT items to a qualified transport company. The company that provided the shipping service was DHL.

Homogeneity and stability of PT items: All larvae and DNAs were individually identified at species level analysing 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". Homogeneity was further ensured by providing all participants aliquots of the same DNA preparations. The stability of the PT items was evaluated by *ad hoc* experiments carried out by EURL-P. Larvae and DNAs preserved in 96% ethanol and stored between -20°C and +20° C maintain their stability up to five years. Quality control required that PTP's staff analyse PT items before distribution.

Distribution of PT items: The PT items were shipped on 17/03/2025, with the deadline for submitting results set for 11/04/2025. Each set of PVI items consisted of 50 ml vial marked with the participant's identification code. Each vial contained four 1.5 ml vials identified with the code assigned by PTP to the PT items. The packaging consisted of a polystyrene and cardboard container containing an adequate number of cooling bricks to ensure that a temperature between 4 and 15°C was maintained inside the package.

Instructions for participants*:* Participants were informed of the shipment date via email on 17/03/2025. The email also contained a link for submitting results, which was active from 17/03/2025 (coinciding with shipment) to 11/04/2025. Participant instructions were made available on the PTP website from 03/02/2025. These instructions also contained information for sending feedback and submitting results. This information was sent to participants along with the PT announcement email and also remarked in the email sent on 17/03/2025 (shipment date).

Data analysis: Feedback and participant results were collected via the online Forms application and transferred from the PTP to an Excel file used for data processing, and further used to generate the tables with the participant results contained in this PT report.

Assigned value: The assigned value was determined by the competence of the staff involved in the analysis and production of the PT items.

Criteria for results evaluation: For each PT item, the result was evaluated by comparing the result reported by the participant with the expected value. Result evaluation in qualitative. Result was 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. PTP evaluated

the result of the participant according to the sensitivity of the method applied. The participant was asked to correctly identify at least the species, for hybrid genomes (i.e. *Anisakis simplex/Anisakis pegreffii* hybrid genotype) the result was evaluated based on the genetic marker used (i.e. mitochondrial vs nuclear markers). Likewise, the correct identification of the subspecies was evaluated according to the resolution of the method applied. Due to the nature of PT items, no statistical parameters was applied for result evaluation. The final evaluation was "positive" if all PT items were correctly identified, otherwise it was "negative".

Confidentiality of results: The confidentiality of this report is guarantee using a unique code that allows the anonymity of participants. The identity of participants is kept confidential and subject to professional secrecy. The EURL-P reserves the right to provide the results of participation in the PT scheme to the competent authorities upon request. The participant will be notified in writing if a competent legislative authority requests the provision of the PT results.

Results provided by participants and performance EVALUATION

Participant code	Applied method	Item code	Result	Assigned value	Outcome	Final evaluation
AMM01	**In house method (PCR and sequencing)**	**AM901** **AM902** **AM903** **AM904**	**NO DNA ISOLATED** ***Anisakis simplex* ss** ***Contracoecum obsculatum*** ***Anisakis pegreffii***	***Pseudoterranova* spp** ***Anisakis simplex* ss** ***Contracoecum obsculatum*** ***Anisakis pegreffii***	**INCORRECT** **CORRECT** **CORRECT** **CORRECT**	**NEGATIVE**
AMM02	EURL-P method 2 (multiplex PCR)	AM905 AM906 AM907 AM908	*Pseudoterranova* spp *Anisakis simplex* *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM03	Published method (PCR and Sequencing)	AM909 AM910 AM911 AM912	*Pseudoterranova decipiens* *Anisakis simplex* *Contracoecum obsculatum B* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM04	EURL-P method 2 (multiplex PCR)	AM913 AM914 AM915 AM916	*Pseudoterranova* spp *Anisakis simplex or A. simplex/pegreffi hybrid* *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM05	**EURL-P method 1 (PCR_RFLP)**	**AM917** **AM918** **AM919** **AM920**	***Pseudoterranova*** ***Anisakis simplex*** **NO AMPLIFICATION** ***Anisakis pegreffii***	***Pseudoterranova* spp** ***Anisakis simplex* ss** ***Contracoecum obsculatum*** ***Anisakis pegreffii***	**CORRECT** **CORRECT** **INCORRECT** **CORRECT**	**NEGATIVE**
AMM06	**EURL-P method 1 (PCR_RFLP)**	**AM921** **AM922** **AM923** **AM924**	**NEGATIVE** ***Anisakis simplex*** ***Contracoecum obsculatum*** ***Anisakis pegreffii***	***Pseudoterranova* spp** ***Anisakis simplex* ss** ***Contracoecum obsculatum*** ***Anisakis pegreffii***	**INCORRECT** **CORRECT** **CORRECT** **CORRECT**	**NEGATIVE**
AMM07	EURL-P method 2 (multiplex PCR)	AM925 AM926 AM927 AM928	*Pseudoterranova* spp *Anisakis simplex* sl *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM08	EURL-P method 1 (PCR_RFLP)	AM929 AM930 AM931 AM932	*Pseudoterranova* spp *Anisakis simplex* *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM09	**EURL-P method 2 (multiplex PCR)**	**AM933** **AM934** **AM935** **AM936**	***Pseudoterranova* spp** ***Anisakis pegreffii*** ***Hysterotilacium aduncum*** ***Anisakis pegreffii***	***Pseudoterranova* spp** ***Anisakis simplex* ss** ***Contracoecum obsculatum*** ***Anisakis pegreffii***	**CORRECT** **INCORRECT** **INCORRECT** **CORRECT**	**NEGATIVE**
AMM10	EURL-P method 2 (multiplex PCR)	AM937 AM938 AM939 AM940	*Pseudoterranova* spp *Anisakis simplex* sl *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM11	EURL-P method 2 (multiplex PCR); In house method (PCR-RFLP and multiplex PCR)	AM941 AM942 AM943 AM944	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE

Participant code	Applied method	Item code	Result	Assigned value	Outcome	Final evaluation
AMM12	Published method (PCR and sequencing)	AM945 AM946 AM947 AM948	*Pseudoterranova decipiens* *Anisakis simplex* *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM13	EURL-P method 1 (PCR_RFLP); EURL-P method 2 (multiplex PCR)	AM949 AM950 AM951 AM952	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM14	EURL-P method 1 (PCR_RFLP); EURL-P method 2 (multiplex PCR); In house method (PCR and sequencing)	AM953 AM954 AM955 AM956	*Pseudoterranova* spp *Anisakis simplex* *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM15	EURL-P method 2 (multiplex PCR)	AM957 AM958 AM959 AM960	*Pseudoterranova* spp *Anisakis simplex or A. simplex/pegreffi hybrid* *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM16	EURL-P method 1 (PCR_RFLP);	AM961 AM962 AM963 AM964	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE
AMM17	EURL-P method 1 (PCR_RFLP)	AM965 AM966 AM967 AM968	*Pseudoterranova* spp (*P. decipiens* ss) *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	*Pseudoterranova* spp *Anisakis simplex* ss *Contracoecum obsculatum* *Anisakis pegreffii*	CORRECT CORRECT CORRECT CORRECT	POSITIVE

Legenda:

- Laboratories that failed the PT are marked in bold.

Summary of results:

Total number of PT panels	17
Number of participants	17
Number of participants that passed the PT	13
Number of participants that failed the PT	4

Overtime comparison of results

Laboratory code 2025	2017	2018	2019	2020	2021	2022	2023	2024	2025
AMM01	NP	NP	NP	POS	POS	POS	**NEG**	POS	**NEG**
AMM02	POS	POS	**NEG**	POS	POS	POS	POS	POS	POS
AMM03	NP	NP	POS	NP	POS	POS	POS	POS	POS
AMM04	NP	NP	NP	NEGA	POS	POS	POS	POS	POS
AMM05	POS	POS	POS	POS	POS	POS	NP	POS	**NEG**
AMM06	POS	POS	POS	**NEG**	POS	POS	**NEG**	POS	**NEG**
AMM07	POS	POS	POS	POS	POS	POS	POS	POS	POS
AMM08	POS	NEGA	NP	NP	POS	POS	**NEG**	POS	POS
AMM09	NP	NP	NP	NP	NP	NP	NP	NP	**NEG**
AMM10	NP	NP	NP	NP	NP	NP	NP	NP	POS
AMM11	POS	POS	POS	POS	POS	POS	POS	**NEG**	POS

Laboratory code 2025	2017	2018	2019	2020	2021	2022	2023	2024	2025
AMM12	NP	NP	NP	NP	NP	NP	NP	NP	POS
AMM13	POS	POS	POS	POS	POS	POS	POS	POS	POS
AMM14	NP	POS	NP	**NEG**	POS	**NEG**	**NEG**	POS	POS
AMM15	NP	POS	NP	NP	NP	NP	**NEG**	POS	POS
AMM16	NP	NP	**NEG**	NP	POS	**NEG**	**NEG**	**NEG**	POS
AMM17	NP	NP	NP	NP	NEGA	POS	**NEG**	POS	POS

NP= no participation. **POS**= positive. **NEG**= negative.

Comments on performance of participants: In the PT round of 2025 16 out of 17 participants received the PT items within 72 hours. The late delivery of the package for one participant was due to custom's delay. Thirteen participants (77%) successfully accomplished the PT, whereas four (23%) failed. In particular, two participants (AMM01 and AMM06) had problems in either DNA extraction and/or DNA amplification from *Pseudoterranova decipiens* larva fragment.
Participant AMM05 was unable to identify *the Contracaecum osculatum* DNA. It can be excluded problem due to the PT item since the same DNA stock was forwarded to all participants. None reported troubles concerning amplification of the DNAs with either EURL-P internal method MI-10 "Identification of Anisakidae Larvae at the species level by multiplex PCR" or EURL-P internal method MI-04 "Identification at species level of parasites of the family Anisakidae by PCR/RFLP". Moreover, the PTP cannot envisage problem with PT items storage during the shipping also according to temperature check reported by the participant (10°C; PT04 Result Form_2025).
Participant AMM09 did not correctly identify the larva fragment of *A. simplex* (it was reported as *A. pegreffii*) and the DNA from *C. osculatum* (it was reported as *Hysterotilacium aduncum*). This participant used the EURL-P internal method MI-10 "Identification of Anisakidae Larvae at the species level by multiplex PCR". The reason for the PT failure could be due to: i) error or inappropriate changes made by the operator during the DNA extraction or PCR amplification steps; ii) DNA contamination during the analysis phase; iii) incorrect interpretation of DNA fragments observed during the electrophoresis run. In addition, the participant has incorrectly reported the "lab code" in the Result Form. The person in charge should take more care on the instruction provided by email concerning where to retrieve the "lab code".

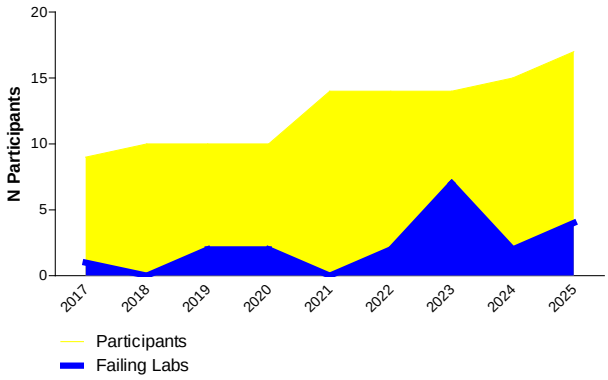

Figure 2. PT07 trend overtime

Performance evaluation if different methods are applied: Concerning the molecular methods applied (Figure 1): i) five participants applied only the EURL-P method MI-04 "Identification at species level of parasites of the family Anisakidae by PCR/RFLP"); ii) seven participants used EURL-P method MI-10 "Identification of Anisakidae Larvae at the species level by multiplex PCR" alone or in addition to in-house method; iii) two participants used both EURL-P methods; iv) two participant used only in house/published methods (one of which was PCR of the EURL-P "MI-04" followed by Sanger sequencing instead of RFLP).

About the use of multiplex PCR (AMM02; AMM10; AMM12; AMM14), a method that doesn't allow to distinguish between *Anisakis simplex* and *A. simplex/pegreffi* hybrid genotype, participants should have been reported in the result both *Anisakis simplex* and *A. simplex / pegreffi hybrid* and not simply *Anisakis simplex*. There was no direct correlation between PT failure and the method used for species identification.

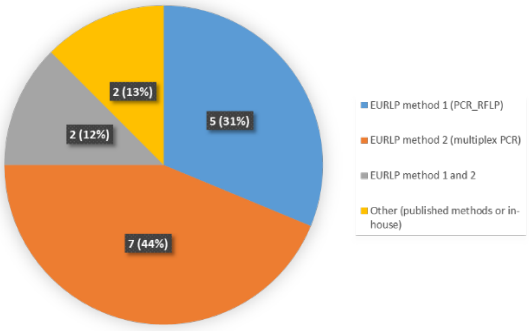

Figure 1. Number of laboratories applying the different methods (percentage are in brackets) for the PT2025

Comments and recommendations based on the outcomes of PT: The reasons for the PT failure were analysed and reported only by two participants. AMM09 reported misreading of the method instructions and incorrect interpretation of the PCR fragment obtained. Noteworthy, this laboratory participated for the first time and did not have reference material for comparison. AMM06 reported a mistake when loading the DNA in the PCR reaction. Following the Individual Report, the participant run again the PCR with the same DNA and the result was identified as *Pseudoterranova* spp., and communicated to PTP. No feedback was received from the other two participants.
Overall, the reason for the PT failure could be due to:

- Errors or inappropriate changes made by the operator during the DNA extraction or PCR amplification steps.
- Exchange of PVI objects during the analysis phase.
- DNA contamination during the analysis phase.
- Incorrect interpretation of DNA fragments observed during the electrophoresis run.
- Incomplete removal of ethanol used to preserve the larvae.
- Errors or inappropriate changes made by the operator during the DNA extraction or PCR amplification steps.
- Use of unsuitable reagents during the DNA extraction or PCR amplification steps.

Compared to the previous years the number of participants slightly increased and three new participants joint the PT. The number of participants that failed the 2025 PT round increased (23%) in comparison to previous years (Figure 2) except for 2023. The overtime comparison indicates that participants that have failed in previous year have the tendency to fail again time by time. However, for some participants, efforts were done to improve the correct application of the molecular identification tests in place.

Written and elaborated by
PTP person in charge

Dr. Marco Lalle

Date 15/01/2026

Verified and issued by
The Director

Dr. A. Casulli

Notes:

1. Personal data are processed in compliance with the regulatory provisions referred to in EU Regulation 2016/679 and Privacy Code, as reported in Legislative Decree no. 101/2018. The data controller of personal data is the Istituto Superiore di Sanità with registered office in Viale Regina Elena n. 299 - 00161 Rome, in the person of its President. In addition, the ISS has appointed its own Data Protection Officer (D.P.O.), e-mail address: responsabile.protezionedati@iss.it. Data are processed exclusively for carrying out the PT activities, for this purpose adequate physical, technical and organizational security measures have been set up to prevent and avoid their destruction and/or loss of integrity, as well as their illicit or incorrect use. Data is accessible only to authorized personnel who has their own credentials and their own operating station. The participant has the rights referred to in art. 15 GDPR et seq., more precisely right of access, right of rectification, right of treatment limitation, right to data portability, right of opposition, as well as the right to lodge a complaint with the Guarantor Authority (art. 77 GDPR and 141 Privacy Code, as reported by Legislative Decree 101/2018). The ISS, in its capacity as Data Controller, undertakes to keep the records of processing activities correctly pursuant to art. 30 GDPR.
2. The original raw data and a copy of Final PT Report are kept for 10 years at the PTP site.
3. Participants may use this report to support their skills to the accreditation body and other interested parties.
4. The accreditation, according to the ISO/IEC 17043 international standard, is regulated by a specific agreement and recognizes the technical competence of the PTP to organize PT schemes. The accreditation body, ACCREDIA (www.accredia.it), does not take any responsibility for the activities related to production of PT items and participants results evaluation.

End of the report