European Union Reference Laboratory for Parasites
Istituto Superiore di Sanità

VIII Proficiency Test on the detection of Anisakis spp. L3 larvae in fish fillets

and

III Proficiency Test on molecular identification of Anisakid nematodes at the species level

Marco Lalle

XIV Workshop of National Reference Laboratories for Parasites
23-24 May, 2019
Istituto Superiore di Sanità

Aim of the PTs

- Identification of the presence of Anisakidae L3 larvae in fish fillets
- Identification of isolated Anisakids larvae/fragments and DNAs by molecular methods (DNA extraction and typing)

- The PTs have been organized following the NRL request during the 2018 EURLP Workshop
- PTs are accredited according to the ISO 17043

PTs time frame

Packages shipped on Monday March 11th

Packages delivery within 72h

PT detection of *Anisakis spp.* L3 larvae in fish fillets

PT performed within 3 days from the samples delivery

Due date to send PT results March 22th

Due date to send Individual PT report April 10th

PT molecular identification of Anisakid nematodes at the species level

PT performed within April 5th

Due date to send PT results April 5th

Due date to send Individual PT report April 19th

Final PT report will be published on EURLP website after the NRL workshop

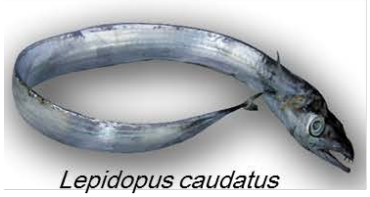
VIII Proficiency Test on the detection of *Anisakis spp.* L3 larvae in fish fillets

Samples and preparation

- A panel of 3 samples (fish fillet sandwiches) has been delivered to each participant
  - 1 Fish fillet sandwiches spiked with 0 Anisakidae larva
  - 1 Fish fillet sandwiches spiked with 1 Anisakidae larva
  - 1 Fish fillet sandwiches spiked with 2 Anisakidae larvae

- Anisakidae L3 larvae were recovered from the body cavity of a heavily parasitized silver scabbardfish from the Mediterranean sea.

- Fillets of farmed rainbow trout were freshly prepared and used to guarantee an Anisakidae-free matrix.

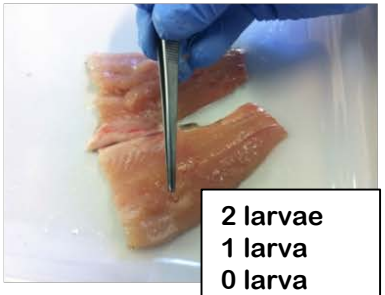


Lepidopus caudatus

Oncorhynchus mykiss irideus

Fillets sealed in plastic bag under vacuum and delivered refrigerated

Pockets were made in the fillets and spiked, or not, with alive L3 larvae

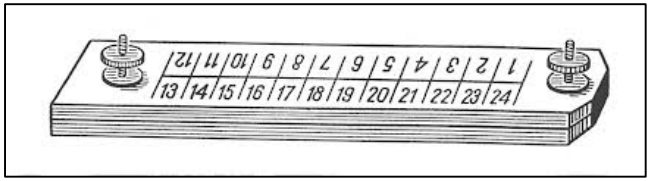


2 larvae
1 larva
0 larva

Detection Methods

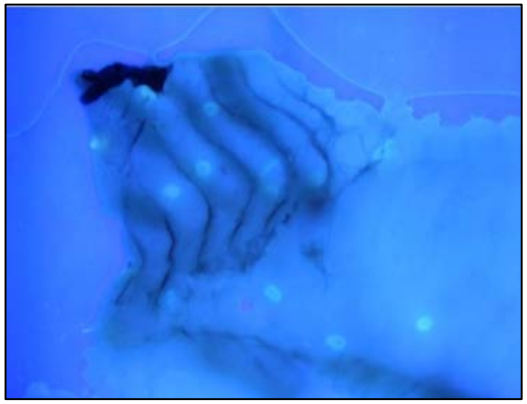
Candling

The laboratories were allowed to use one (or a combination) of the following methods :



Compressorium

Artificial digestion



UV examination after freezing

A step by step protocol for each method was supplied and any deviation from the main protocol should be reported!

Evaluation criteria

The PT evaluation is only qualitative (presence or absence of larvae). Due to low number of samples and size of the larvae, no statistical analysis of the results is applied.

The result is "correct" if the laboratory detected Anisakidae larvae in the three spiked samples
The result is "incorrect" if the laboratory did not detect any larva in the spiked samples.

The PT is considered "positive" if no "incorrect" results were obtained; the PT is considered "negative" if at least one "incorrect" result was obtained.

PT Participants

26 Participants:
23 NRLs
3 Public labs
21 MS +2 non MS

first time participant

Results-1

Lab code	N° of spiked/detected larvae			Method(s)	Final Evaluation
	0	1	2		
A1	0	2	1	D	positive
A2	0	1	2	D	positive
A3	0	1	3	D	positive
A5	0	1	1	D	positive
A6	0	1	2	C+D	positive
A7	0	1	3	C+UV+D	positive
A10	0	1	2	D	positive
A11	0	1	2	D	positive
A12	0	1	2	D	positive
A13	0	1	2	D	positive
A15	0	1	1	C+D	positive
A16	0	1	2	UV	positive
A17	0	1	2	UV	positive
A18	0	1	2	D	positive
A19	0	0	2	D	**negative**
A20	0	1	2	C+Co+D	positive
A21	0	1	2	D	positive
A23/A33/A25	0	1	1	D	positive
A26	0	1	2	D	positive
A28	0	1	2	UV	positive
A29	0	1	2	UV	positive
A30	0	1	2	D	positive
A31	0	0	2	C+D	positive
A35	0	1	2	D	**negative**
A36	0	1	2	D	positive
A39	0	1	2	UV	positive

C: Candling
Co: *Compressorium*
UV: UV ex. after freezing
D: Digestion

Results-2

Participation

26/26 labs sent the results

Method

- **16** Artifificial digestion alone
- **4** Artificial digestion in combilnation with Candling (4)
- **1** UV+D+Candling
- **5** UV alone

Labs reported changes in the digestion method:

- **10** "chopped" the fish fillets by hands, knives or tweezers
- **1** "chopped" the fish fillets by stomacher, perform digestion in TrichinEasy apparatus at 35-37°C.
- **1** used an internal SOP for which digestion was done in a 2L glass beaker using 0.2 % HCL and 0,5 % liquid pepsin in a final volume of 1.5L at 35°C for 1h.
- **1** performed the digestion at 42°C

Detection

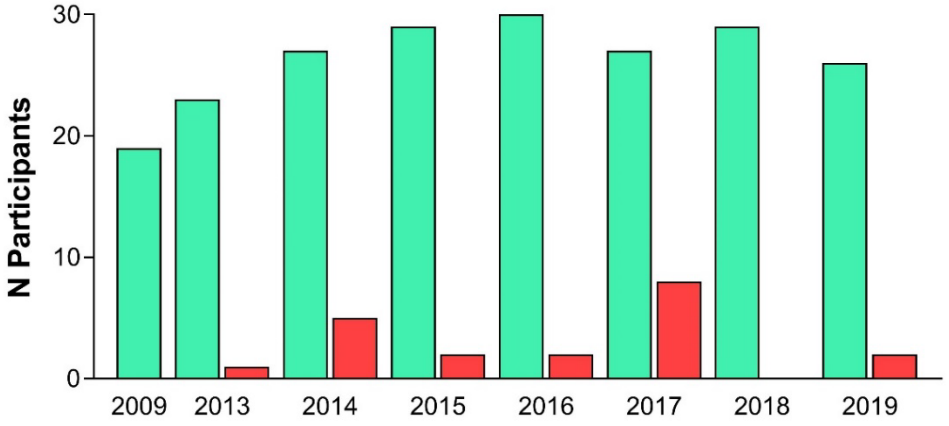
- **2 laboratory failed to pass the PT reporting false negatives**
- **3 laboratories overestimate the samples**
- **3 laboratories underestimate the sample with 2 larvae**

PT Trend

Laboratory code	2014	2015	2016	2017	2018	2019
A1	P	P	P	P	P	P
A2	P	P	P	N	P	P
A3	N	P	P	P	P	P
A5	P	P	P	P	P	P
A6	P	P	P	P	P	P
A7	P	P	P	P	P	P
A8	P	P	P		P	
A9	P		P	N	P	
A10	P	P	N	P	P	P
A11	P	N	P	N	P	P
A12	P	N	P	P	P	P
A13	P	P	P	N	P	P
A15	P	P	P	P	P	P
A16	N	P	P	N	P	P
A17	*NR*		P	P	P	P
A18	P	P	P	P	P	P
A19	P	P	P	P	P	N
A20	P	P	P	P	P	P
A21	P	P	P	P	P	P
A23/A33/A25*	N	P	P	N	P	P
A25	P	P	P	N		
A26	P	P	P	P	P	P
A28	P		P	P	P	P
A29			P		P	P
A30		P	P	P	P	P
A31		P	P	P	P	P
A35					P	N
A36				P	P	P
A37					N	
A38					P	
A39						P

PT panel

2009: 1 sample
2013: 3 samples (naturally infected fish)
2014-2019: 3 samples (farmed fish)



Conclusions

VII PT on the detection of Anisakis spp. L3 larvae in fish fillets

- Over the last years, the number of PT participants is declining
- The overall performance of laboratory is stable and failures are due to novel unexperienced personnel

The highly over/underestimation of the number of larvae (8/26) reported is remarkable and mainly associated with the use of artificial digestion.

As for previous years, lack of routine in the analysis of fish samples parasitized by Anisakidae has, generally, a negative impact on the PT performance.

- Low sensitive methods, compressorium and candling, are still used but exclusively in combination with artificial digestion or UV method. This is a step forward!!

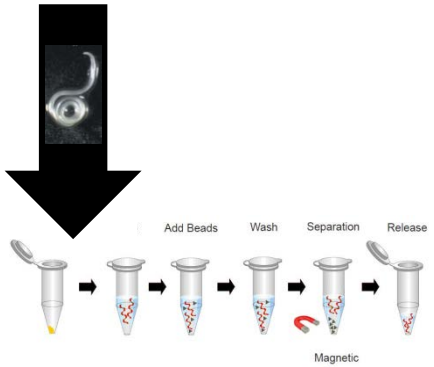
- The relative percentage of detection methods adopted did not change substantially over the last years, although more labs use now UV press method.

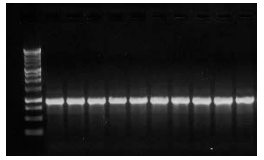
- Artificial digestion still remain the method of choice! Largely because it doesn't require any special equipment.

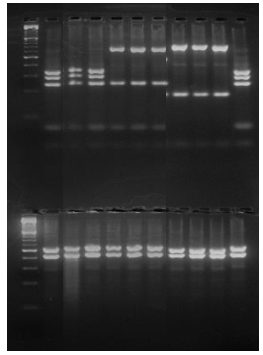
- UV-press method is applied largely in specialized laboratories that perform routine inspection of fish samples

III Proficiency Test on molecular identification of Anisakidae nematodes at the species level

Samples preparation







A panel of 4 samples has been delivered to each participant

- 2 tubes containing a single fragment of Anisakidae L3 larva each *(P. decipiens sl; A. simplex ss)*
- 2 tubes containing DNA extracted from a single Anisakidae L3 larva *(A. pegreffii; P. decipiens sl)*

Larvae were collected from body cavity of infected fishes*

All larvae have been individually identified at species level by analyzing one of their fragments by the EURLP method "Identification at species level of parasites of the family Anisakidae by PCR/RFLP"

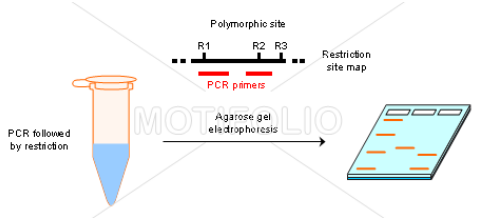
The DNAs have been extracted from single larvae and also identified at species level by the above method. Homogeneity is ensured by providing to all participants aliquots of the same DNA preparations.

*We are grateful to Ute Ostermeyer (Max Rubner-Institut, Germany) and Arne Levsen (Institute of Marine Research, Norway) for providing A. simplex and P. decipiens larvae.

Detection Methods

"Identification at species level of parasites of the family Anisakidae by PCR/RFLP"

rDNA-ITS

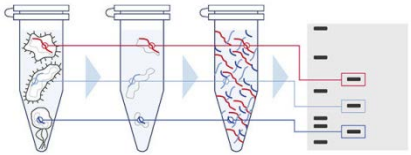


A. pegreffii
A. simplex ss
A. simplex/pegreffii Hybrid
A. simplex C
A. ziphidarium
A. physeteris
A. typica
A. sp A
Pseudoterranova sl (P. decipiens s.s.)
Hysterotilacium spp (H. aduncum)
Contracaecum rudolphii (A, B, C)

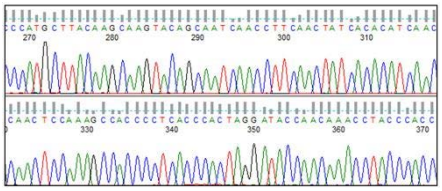
A. pegreffii
A. simplex s.l. (incl. A. simplex/pegreffii hybrid)
A. Physeteris (incl. A. brevispiculata and A. paggiae)
A. typica
Pseudoterranova sl (P. decipiens s.s.)
Hysterotilacium spp (H. aduncum)
Contracaecum rudolphii (A, B, C)

"Identification of Anisakidae Larvae at the species level by multiplex PCR"

ITS



Any other suitable molecular method performed by the participant laboratory (i.e. PCR and sequencing)



- **Applied method to be describe in the Form 3 (MO/POPVI-00/03.07: Procedure)**

- **List the instruments, reagents and materials used to perform the test to be describe in the Form 2 (MO/POPVI-00/02.07: List of instruments, reagents and materials used to perform the test).**

- **In case you used a published method, indicate the reference and any variation from the original procedure in the dedicated column.**

Evaluation criteria

The PT evaluation is only **qualitative** and no statistical analysis of the results is applied.

The result is "correct" if PT items are correctly identified.
The result is "incorrect" if PT items are incorrectly identified

The PT is considered "positive" if no "incorrect" results were obtained; the PT is considered "negative" if at least one "incorrect" result was obtained.

PT Participants

10 Participants:
10 NRLs
1 Non MS

first time participant

Results-1

Laboratory code	N° of samples correctly identified		N° of samples NOT correctly identified		Method(s)	Final evaluation
	Larva	DNA	Larva	DNA		
A3	1	1	1	1	PR (EURLP)	Negative
A6	2	2	0	0	PM (EURLP)	Positive
A7	2	2	0	0	PR+PM (EURLP)	Positive
A10	2	2	0	0	PR (EURLP)	Positive
A12	2	2	0	0	PR (EURLP)	Positive
A16	2	2	0	0	PM (EURLP)	Positive
A17	2	2	0	0	PS	Positive
A20	2	2	0	0	PR (EURLP)	Positive
A28	1	2	1	0	PS	Negative
A39	2	2	0	0	modified PM	Positive

PR=PCR-RFLP
PM=PCR Multiplex
PS=PCR+Sequencing

Results-2

Participation

10/10 labs sent the results

Method

- **1** PCR-RFLP and PCR Multiplex
- **4** PCR-RFLP
- **3** PCR Multiplex
- **2** PCR+Sequencing (1 Cox2 gene and 1 ITS)

Method deviation from the suggested

- **5** used different DNA extraction kits and gel staining
- **4** reported different Taq polymerases

Detection

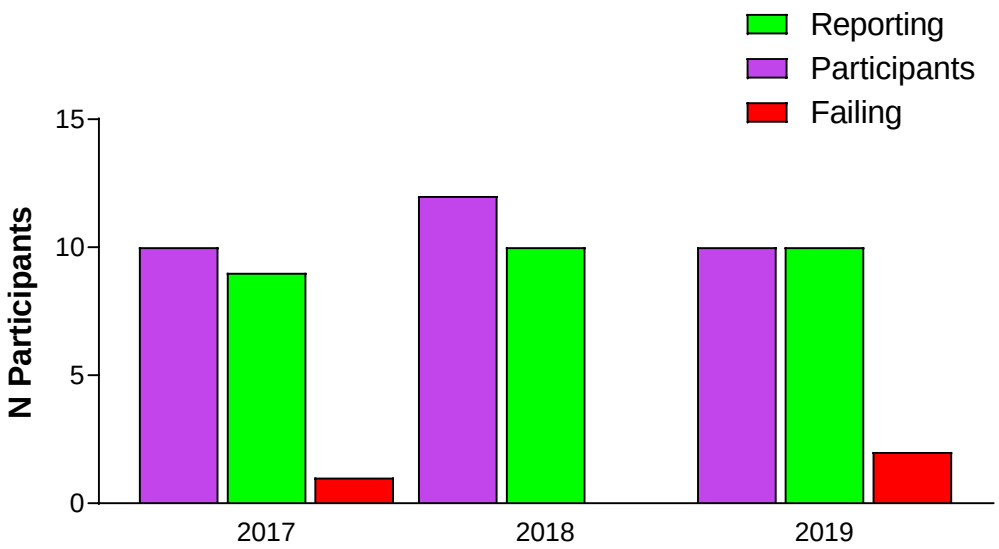
2 labs failed to pass the PT.

One reported problem in DNA extraction from 1 larva.

One reported incorrect identification of one larva sample and one DNA sample

PT trend

Laboratory code	2017	2018	2019
A1	NA	-	-
A3	-	-	N
A6	P	P	P
A7	P	P	P
A8	-	P	-
A10	P	P	P
A11	-	NA	-
A12	P	P	P
A16	P	P	P
A17	N	P	P
A20	P	P	P
A28	P	P	N
A31	P	NA	-
A38	-	P	-
A39	-	-	P



Conclusions

III PT on molecular identification of Anisakid nematodes at the species level

- All the laboratories provided results.
- Two laboratories failed the PT due to unexperienced personnel.
- Compared to the previous year two new laboratories participated.
- PT performance was not affected by the applied method.
- This PT round underlined the importance to extensively train new personnel joining the lab.

Thank you for your attention!