Report of the 4rd NRL proficiency testing on "Detection of Anisakidae L3 larvae in fish fillets"

March - April, 2015

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1 Introduction

Nematode worms of the Anisakidae family are zoonotic parasites circulating in most marine fish and cephalopods at the larval stage, and in marine mammals at the adult stage (Pozio, 2013). Humans acquire the infection by the consumption of raw, undercooked or marinated fish (Audicana and Kennedy, 2008). According to the Commission Regulation (EC) No 853/2004, food business operators must ensure that fishery products have been subjected to a visual examination for the purpose of detecting visible parasites before being placed on the market. They must not place on the market fishery products for human consumption that are obviously contaminated with parasites.

2 Scope

The PT aims to evaluate the competence of the appointed NRLs to detect Anisakidae larvae in fish fillets. The organization of proficiency testings (PTs) falls in the duties of the EURL for Parasites, as it is stated in the Regulation (EC) No 882/2004 of the European Parliament and of the Council (Commission Regulation No. 882/2004). The 4th PT on detection of Anisakidae larvae in fish fillets has been organized following the 3rd PT in 2014 and in agreement to the request of NRLs during the 2014 EURLP annual workshop.

In 2015, the PT on "Detection of Anisakidae L3 larvae in fish fillets" has been also accredited by Italian Accreditation Body (Accredia) according to the ISO standard 17043.

3 Time frame

The PT was first announced to NRLs by email on January 15th, 2015. A second email was sent to all NRLs on February 6th, 2015 including the participation form (see Annex 1; MO/MAQPVI/01.PT Request Form) and the deadline to send it back. On March 16th, 2015, the samples were dispatched to participants by an international courier. Reporting deadline was 25th March, 2015.

4 Test material

The test material forwarded to each laboratory consisted of a panel of 3 fish fillets sandwiches containing in the core a different number of third stage Anisakidae larvae (L3). Farmed rainbow trout fillets (*Oncorhynchus mykiss irideus*) were used to guarantee fish matrix free from L3 infestation sandwiches. The panel includes a negative sample, one sample spiked with a single L3 and one sample spiked with three L3. Live L3 larvae of *Anisakis pegreffii,* free of their capsule, were recovered from the body cavity of heavily parasitized silver scabbardfish (*Lepidopus caudatus*) from the Mediterranean sea and used to spike samples (Figure 1 and 2, Annex 2). L3 were identified at genus level by microscopic examination and a pool of larvae were identified at species level by PCR-RFLP of the ITS region according to the EURLP accredited method MI-04. L3 larvae were carefully transferred by tweezers in pockets made in the middle of fish fillets (Figure 3, Annex 2). Fish sandwiches were then put individually in a plastic bag sealed under vacuum, identified by an alphanumeric code stuck on the plastic bag, without any indication of the level of contamination or any information on the identity of the testing laboratory, and stored at +4 °C (Figure 4, Annex 2). The same code with the number of larvae was reported in an Excel file. Samples were forwarded to each participant laboratory by an international courier in a polystyrene box with cooler packages to maintain a temperature lower than +15 °C during shipping. Packages were forwarded according to the international forwarding regulations. The stability of the samples in the package has been evaluated by ad hoc experiments made at EURLP. Larvae in samples sealed under vacuum and stored between +4 °C and +15 °C, survive at least 7 days from the date of preparation.

5 Instructions to participants

From February 6th, 2015, general information on the PT aim, instructions and procedures were made available to NRLs on the EURLP web site (http://www.iss.it/crlp/index.php?lang=2&anno=2015&tipo=28).

Electronic copies of the forms, which had to be filled in and sent back to EURLP, were sent by e-mail on March 16th, 2015, (see Annex 1; MO/POPVI/00 from 01 to 04):

1) "Check package", to inform about the content and the condition of the PT material at arrival in the lab;
2) "Instruments and Materials List" needed to perform the suggested methods;
3) "Procedure", step by step description of the four suggested methods;
4) "Result", to report the number of larvae detected in each sample.

The code assigned to the laboratory was sent individually to each participant laboratory by a separate e-mail on March 16th 2015.

To make comparable the results, all participant laboratories should follow the protocol step by step or, if the case, describe any variation. Four tests were suggested and described: 1. candling; 2. compressorium; 3. UV examination after freezing; and 4. artificial digestion.

6 Criteria for result evaluation and statistical analysis

The evaluation was only qualitative (presence or absence of larvae). The result was "correct" if the laboratory detected Anisakidae larvae in the two spiked samples and no larva in the non-spiked sample. The result was "incorrect" if the laboratory did not detect any larva in the spiked samples or reported a false positive. The PT was considered "positive" if no "incorrect" results were obtained; the PT was considered "negative" if at least one "incorrect" result was obtained.

Due to the low number of samples in the panel, no statistical analysis of the results was performed. On 30th of March 2015, the EURLP provided an Individual PT Reports (see Annex 3), which was sent by email to each participant laboratory as a pdf file. The individual PT Report included: i) the number of spiked larvae per sample; ii) the number of larvae detected in each sample by each participant laboratory; iii) the final evaluation; and iv) recommendation based on the laboratory performance.

The EURLP also provided a Final PT Report, including results obtained by all participant laboratories. The final report was presented at the NRL workshop and published on the EURLP website (see Annex 3). To guarantee confidentiality in the final report, participant laboratories were identified only by an alphanumeric code.

7 Participating laboratories

Twenty-seven laboratories agreed to participate in the PT, including 25 NRLs (two NRLs from Finland), globally representing 24 MS and one non-EU Country. Portugal and Bosnia and Herzegovina participated in the PT for the first time (Annex 4).

8 Results

8.1 Delivery of the package to NRLs

All packages were delivered to participant laboratories within 24-72 hours (1 day for 18 labs, 2 days for 8 labs, and 3 days for 1 lab) (see Annex 6). At delivery, the internal temperature of packages was ≤ 10 °C in 26 parcels (95%) and 12 °C in one. The elapsed time from package delivery at the NRL and its control was ≤ 1 h for 26 laboratories and approximately 12 h for 1 participant.

8.2 Test methods

Seventeen laboratories used the digestion method only, five laboratories used first the UV, candling or compressorium and then the digestion method (see Annex 6). One labs used the UV

method only, one lab used candling followed by UV method, one lab used the compressorium only, and one lab used the candling only.

Significant deviations from the suggested protocols were reported by six participants only for the digestion method as follow: two labs chopped fish fillets (Form 3, 1.4) by stomacher; one lab performed digestion at 40-43 °C (Form 3, 1.1); one lab followed an internal SOP for which digestion is done in a 2L glass beaker (Form 3, 1.2) using 0.2 % HCl and liquid pepsin in a final volume of 1.5L (Form 2, 5.3 and 5.4; Form 3, 1.1) at 30 °C for 2 h (Form 3, 1.7; 1.8). One laboratory also performed molecular analysis of the recovered larvae by an accredited PCR-RFLP method. Larvae were typed as *A. pegreffii*.

8.3 Evaluation

All participant labs sent the results. One lab, which tested the PT samples by candling, detected motile larvae. Two (7.4%) out of 27 participant laboratories failed to pass the PT. In one case, the laboratory did not find any larva in the sample spiked with one larva, and in one case the laboratory reported a false positive and a false negative. The misdetection could be due to lack of experience of the analysts to perform the digestion method and in the other case it could be due to sample shuffling.

8.4 Corrective Actions

According to the Commission request, corrective actions were planned for the two laboratories, which failed the PT. An e-mail was sent to these laboratories, on March 30th 2015, asking them to carefully check the possible reason(s) of the failure and to send back their comments filling in the Corrective Action Participation form sent by email. No lab sent a feedback reporting the cause of the PT failure. These labs were also informed that a second panel of samples would be sent on April 13th, 2015. Two other NRL laboratories, which did not participate to the fourth PT round, asked to be included in the panel forwarding for the corrective action. All the four participating labs were informed that the new panel was part of a corrective action for those labs, which failed the 2015 annual PT round. This corrective action was considered as an External Quality Assessment (EQA) scheme, therefore only an Individual Report was provided to each participant.

The deadline to send the signed participation form was April 7th, 2015. Sample preparation was as reported in paragraph 4. On April 13th, 2015, samples were dispatched to participants by an international courier. Reporting deadline was April 22nd, 2015. The procedure and applicable methods were the same as detailed for the PT, and the 4 Forms and the individual lab codes were sent individually to each participant laboratory by a separate email on April 13th, 2015.

Delivery of the package to NRLs, test methods used, and results are summarized in Annex 7. Three laboratories received the samples within 24 h and 1 lab within 48 h. At delivery, the internal temperature of all packages was ≤ 10 °C in 3 parcels, and one lab reported “cool” temperature.

Two laboratories used the digestion method only, 1 lab used the UV method only, and one lab used the UV method and the digestion method. One lab reported deviation in the digestion method following an internal SOP for which digestion is done using 0.2 % HCl and liquid pepsin (Form 2, 5.3 and 5.4; Form 3, 1.1) at 35 °C (Form 3, 1.7; 1.8).

The evaluation was only qualitative (presence or absence of larvae) as detailed in the paragraph 6. All labs correctly identified all samples and passed the EQA. An individual report was sent to each laboratory by email on April 22nd, 2015.

9 Conclusions

In agreement with previous PTs, these results suggest that most of participating laboratories have a high level of competence in the identification of Anisakidae larvae, irrespective of the detection method used. The artificial digestion is still the most used method, followed by UV examination after freezing, which is often used in combination with the previous method.

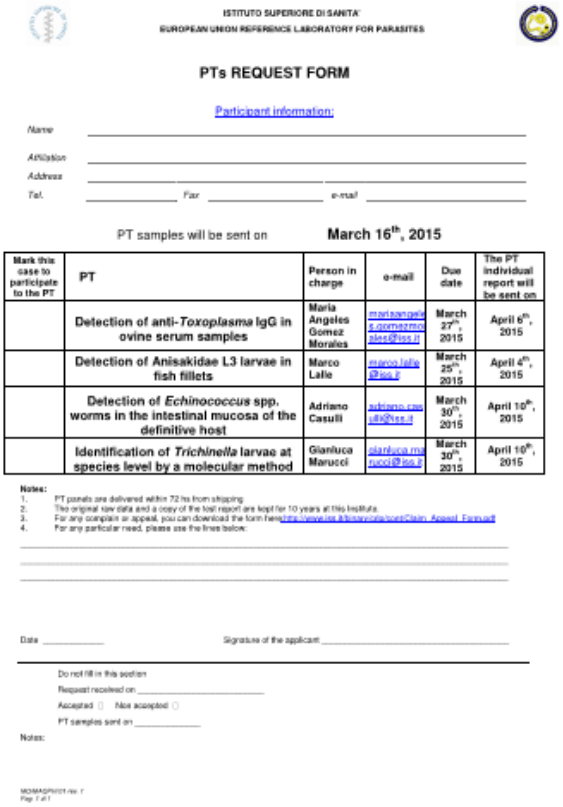
10 References

Pozio E. 2013. Integrating animal health surveillance and food safety: the example of Anisakis. Rev Sci Tech 32, 487-496.

Audicana MT, Kennedy MW. 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin Microbiol Rev 21, 360-379.

Method MI-04: "Identification at species level of parasites of the family Anisakidae by PCR/RFLP" (http://www.iss.it/binary/crlp/cont/MI_04_website_EN.pdf)

Annex 1



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EUROPEAN UNION REFERENCE LABORATORY FOR PARASITES

PTs REQUEST FORM

Participant information:

Name _____

Affiliation _____

Address _____

Tel. _____ Fax _____ e-mail _____

PT samples will be sent on **March 16th, 2015**

Mark this case to participate to the PT	PT	Person in charge	e-mail	Due date	The PT individual report will be sent on
	Detection of anti-*Toxoplasma* IgG in ovine serum samples	Maria Angeles Gomez Morales	mariaangeles.gomezmorales@iss.it	March 27th, 2015	April 6th, 2015
	Detection of Anisakidae L3 larvae in fish fillets	Marco Lalle	marco.lalle@iss.it	March 25th, 2015	April 4th, 2015
	Detection of *Echinococcus* spp. worms in the intestinal mucosa of the definitive host	Adriano Casulli	adriano.casulli@iss.it	March 30th, 2015	April 10th, 2015
	Identification of *Trichinella* larvae at species level by a molecular method	Gianluca Marucci	gianluca.marucci@iss.it	March 30th, 2015	April 10th, 2015

Notes:
1. PT panels are delivered within 72 hs from shipping
2. The original raw data and a copy of the test report are kept for 10 years at this Institute
3. For any complaint or appeal, you can download the form here: http://www.iss.it/binaries/site/cont/Claim_Appeal_Form.pdf
4. For any particular need, please use the lines below:

_____

_____

_____

Date _____ Signature of the applicant _____

Do not fill in this section

Request received on _____

Accepted ☐ Not accepted ☐

PT samples sent on _____

Notes:

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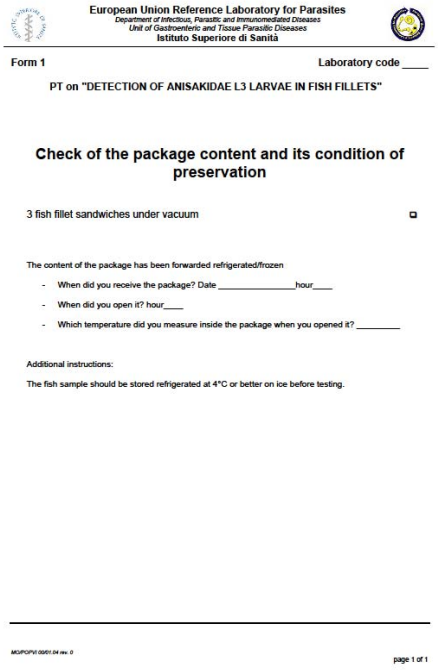

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Form 1 **Laboratory code ____**

PT on "DETECTION OF ANISAKIDAE L3 LARVAE IN FISH FILLETS"

Check of the package content and its condition of preservation

3 fish fillet sandwiches under vacuum ☐

The content of the package has been forwarded refrigerated/frozen

- When did you receive the package? Date _____hour____
- When did you open it? hour____
- Which temperature did you measure inside the package when you opened it? _____

Additional instructions:

The fish sample should be stored refrigerated at 4°C or better on ice before testing.

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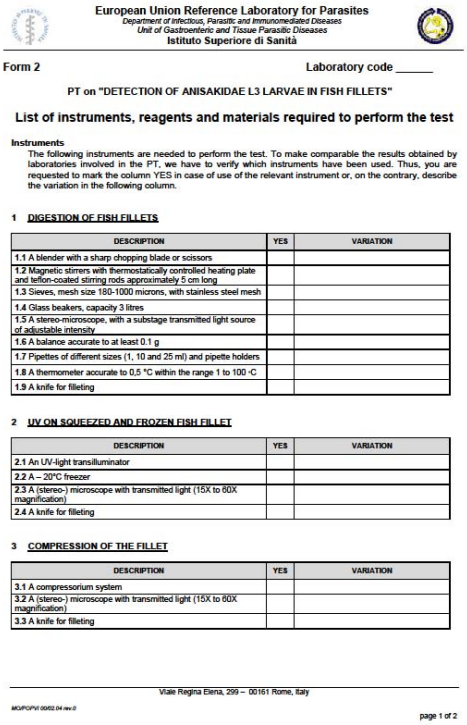

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Form 2 **Laboratory code _____**

PT on "DETECTION OF ANISAKIDAE L3 LARVAE IN FISH FILLETS"

List of instruments, reagents and materials required to perform the test

Instruments

The following instruments are needed to perform the test. To make comparable the results obtained by laboratories involved in the PT, we have to verify which instruments have been used. Thus, you are requested to mark the column YES in case of use of the relevant instrument or, on the contrary, describe the variation in the following column.

1 DIGESTION OF FISH FILLETS

DESCRIPTION	YES	VARIATION
1.1 A blender with a sharp chopping blade or soissors		
1.2 Magnetic stirrers with thermostatically controlled heating plate and teflon-coated stirring rods approximately 5 cm long		
1.3 Sieves, mesh size 180-1000 microns, with stainless steel mesh		
1.4 Glass beakers, capacity 3 litres		
1.5 A stereo-microscope, with a substage transmitted light source of adjustable intensity		
1.6 A balance accurate to at least 0.1 g		
1.7 Pipettes of different sizes (1, 10 and 25 ml) and pipette holders		
1.8 A thermometer accurate to 0.5 °C within the range 1 to 100 -C		
1.9 A knife for filleting		

2 UV ON SQUEEZED AND FROZEN FISH FILLET

DESCRIPTION	YES	VARIATION
2.1 An UV-light transilluminator		
2.2 A – 20°C freezer		
2.3 A (stereo-) microscope with transmitted light (15X to 60X magnification)		
2.4 A knife for filleting		

3 COMPRESSION OF THE FILLET

DESCRIPTION	YES	VARIATION
3.1 A compressorium system		
3.2 A (stereo-) microscope with transmitted light (15X to 60X magnification)		
3.3 A knife for filleting		

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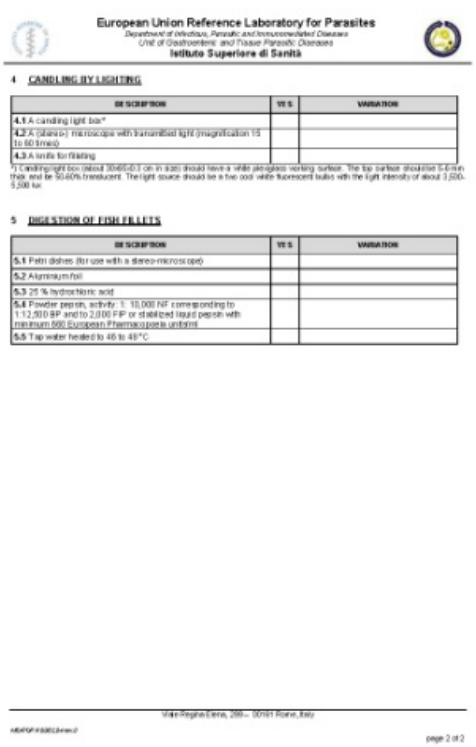

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4 CANDLING BY LIGHTING

DESCRIPTION	YES	VARIATION
4.1 A candling light box*		
4.2 A (stereo-) microscope with transmitted light (magnification 15 to 60 times)		
4.3 A knife for filleting		

*) Candling light box (about 30x60x0.3 cm in size) should have a white plexiglass working surface. The top surface should be 5-6 mm thick and be 50-60% translucent. The light source should be a two cool white fluorescent bulbs with the light intensity of about 3,500-5,500 lux.

5 DIGESTION OF FISH FILLETS

DESCRIPTION	YES	VARIATION
5.1 Petri dishes (for use with a stereo-microscope)		
5.2 Aluminium foil		
5.3 25 % hydrochloric acid		
5.4 Powder pepsin, activity 1: 10,000 NF corresponding to 1:12,500 BP and to 2,000 FIP or stabilized liquid pepsin with minimum 600 European Pharmacopoeia units/ml		
5.5 Tap water heated to 45 to 48°C		

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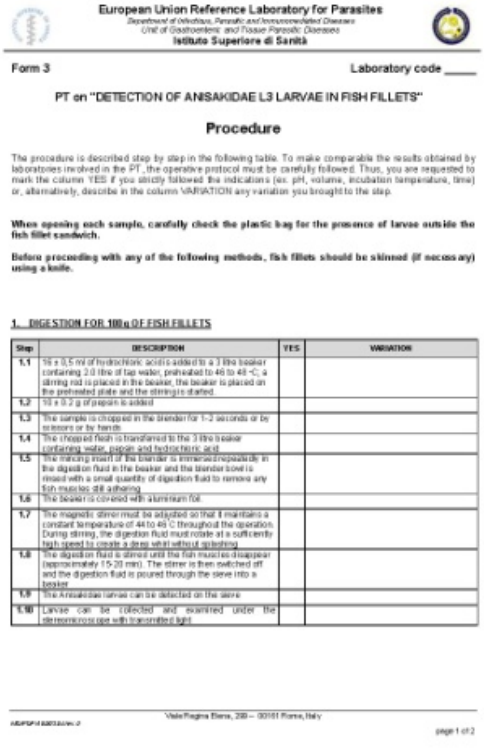

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Form 3 Laboratory code ____

PT on "DETECTION OF ANISAKIDAE L3 LARVAE IN FISH FILLETS"

Procedure

The procedure is described step by step in the following table. To make comparable the results obtained by laboratories involved in the PT, the operative protocol must be carefully followed. Thus, you are requested to mark the column YES if you strictly followed the indications (ex. pH, volume, incubation temperature, time) or, alternatively, describe in the column VARIATION any variation you brought to the step.

When opening each sample, carefully check the plastic bag for the presence of larvae outside the fish fillet sandwich.

Before proceeding with any of the following methods, fish fillets should be skinned (if necessary) using a knife.

1. DIGESTION FOR 100 g OF FISH FILLETS

Step	DESCRIPTION	YES	VARIATION
1.1	10 ± 0,5 ml of hydrochloric acid is added to a 3 litre beaker containing 2.0 litre of tap water, preheated to 46 to 48 °C; a stirring rod is placed in the beaker, the beaker is placed on the preheated plate and the stirring is started		
1.2	10 ± 0.2 g of pepsin is added		
1.3	The sample is chopped in the blender for 1-2 seconds or by scissors or by hands		
1.4	The chopped flesh is transferred to the 3 litre beaker containing water, pepsin and hydrochloric acid		
1.5	The mincing insert of the blender is immersed repeatedly in the digestion fluid in the beaker and the blender bowl is rinsed with a small quantity of digestion fluid to remove any fish muscles still adhering		
1.6	The beaker is covered with aluminium foil		
1.7	The magnetic stirrer must be adjusted so that it maintains a constant temperature of 44 to 46 C throughout the operation. During stirring, the digestion fluid must rotate at a sufficiently high speed to create a deep whirl without splashing		
1.8	The digestion fluid is stirred until the fish muscles disappear (approximately 1.5-20 min). The stirrer is then switched off and the digestion fluid is poured through the sieve into a beaker		
1.9	The Anisakidae larvae can be detected on the sieve		
1.10	Larvae can be collected and examined under the stereomicroscope with transmitted light		

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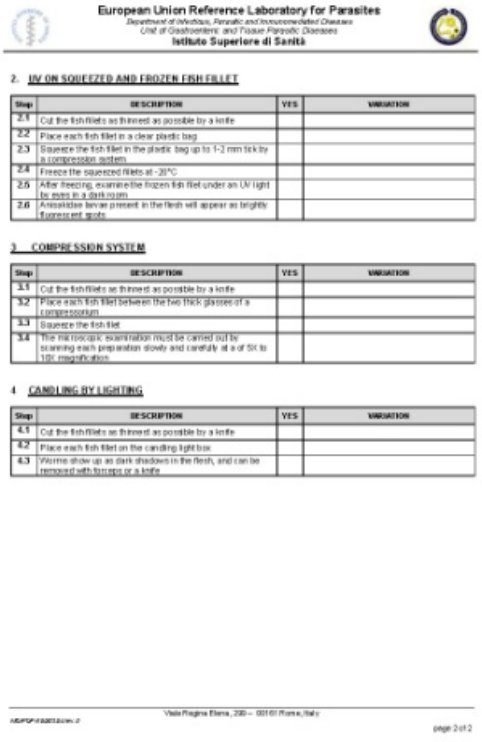

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2. UV ON SQUEEZED AND FROZEN FISH FILLET

Step	DESCRIPTION	YES	VARIATION
2.1	Cut the fish fillets as thinnest as possible by a knife		
2.2	Place each fish fillet in a clear plastic bag		
2.3	Squeeze the fish fillet in the plastic bag up to 1-2 mm tick by a compression system		
2.4	Freeze the squeezed fillets at -20°C		
2.5	After freezing, examine the frozen fish filet under an UV light (in eyes in a dark room)		
2.6	Anisakidae larvae present in the flesh will appear as brightly fluorescent spots		

3. COMPRESSION SYSTEM

Step	DESCRIPTION	YES	VARIATION
3.1	Cut the fish fillets as thinnest as possible by a knife		
3.2	Place each fish fillet between the two thick glasses of a compressorium		
3.3	Squeeze the fish filet		
3.4	The macroscopic examination must be carried out by scanning each preparation slowly and carefully at a of 5X to 10X magnification		

4 CANDLING BY LIGHTING

Step	DESCRIPTION	YES	VARIATION
4.1	Cut the fish fillets as thinnest as possible by a knife		
4.2	Place each fish fillet on the candling light box		
4.3	Worms show up as dark shadows in the flesh, and can be removed with forceps or a knife		

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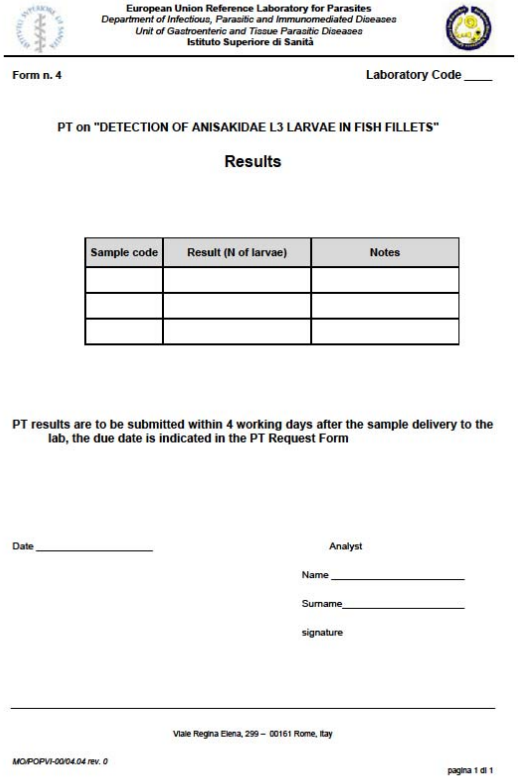

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Form n. 4 Laboratory Code ____

PT on "DETECTION OF ANISAKIDAE L3 LARVAE IN FISH FILLETS"

Results

Sample code	Result (N of larvae)	Notes

PT results are to be submitted within 4 working days after the sample delivery to the lab, the due date is indicated in the PT Request Form

Date _____ Analyst

Name _____

Surname _____

signature

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Annex 2

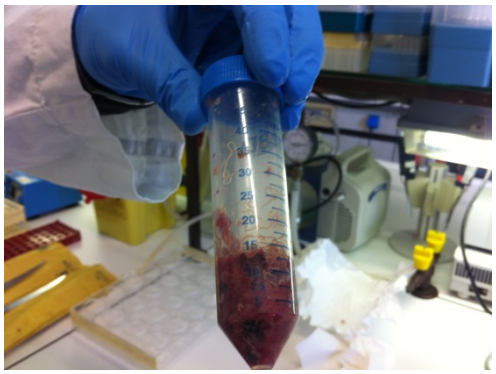


Figure 1. Viscera of silver scabbardfish parasitized by Anisakidae L3 larvae

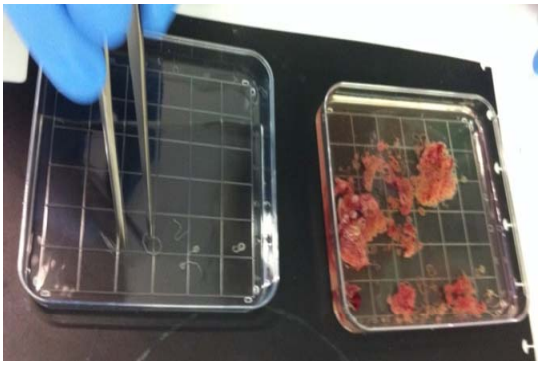


Figure 2. Recovery of Anisakidae L3 larvae

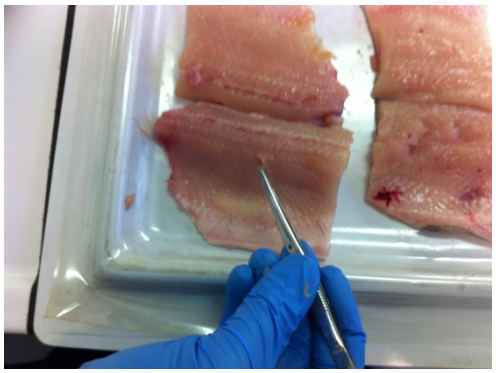
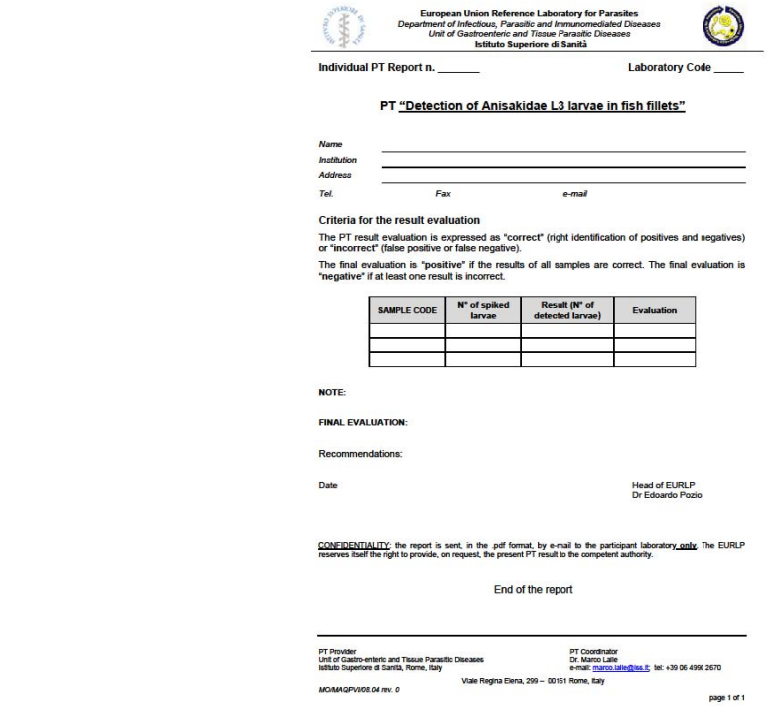


Figure 3. L3 larvae are transferred by tweezers in pockets made in the middle of fish fillets

Figure 4. Fish fillets sandwiches are packed under vacuum

Annex 3



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Individual PT Report n. _____ **Laboratory Code _____**

PT "Detection of Anisakidae L3 larvae in fish fillets"

Name _____
Institution _____
Address _____
Tel. *Fax* *e-mail*

Criteria for the result evaluation

The PT result evaluation is expressed as "correct" (right identification of positives and negatives) or "incorrect" (false positive or false negative).

The final evaluation is "positive" if the results of all samples are correct. The final evaluation is "negative" if at least one result is incorrect.

SAMPLE CODE	N° of spiked larvae	Result (N° of detected larvae)	Evaluation

NOTE:

FINAL EVALUATION:

Recommendations:

Date

Head of EURLP
Dr Edoardo Pozio

CONFIDENTIALITY: the report is sent, in the .pdf format, by e-mail to the participant laboratory only. The EURLP reserves itself the right to provide, on request, the present PT result to the competent authority.

End of the report

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Istituto Superiore di Sanità, Rome, Italy

PT Coordinator
Dr. Marco Lalle
e-mail: marco.lalle@iss.it; tel: +39 06 4990 2670

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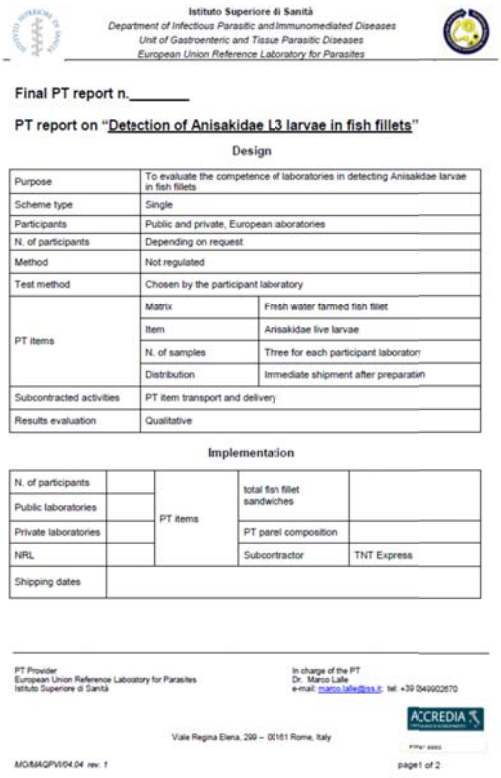

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Final PT report n._____

PT report on "Detection of Anisakidae L3 larvae in fish fillets"

Design

Purpose	To evaluate the competence of laboratories in detecting Anisakidae larvae in fish fillets	
Scheme type	Single	
Participants	Public and private, European laboratories	
N. of participants	Depending on request	
Method	Not regulated	
Test method	Chosen by the participant laboratory	
PT items	Matrix	Fresh water farmed fish fillet
	Item	Anisakidae live larvae
	N. of samples	Three for each participant laboratory
	Distribution	Immediate shipment after preparation
Subcontracted activities	PT item transport and delivery	
Results evaluation	Qualitative	

Implementation

N. of participants		PT items	total fish fillet sandwiches	
Public laboratories				
Private laboratories			PT panel composition	
NRL			Subcontractor	TNT Express
Shipping dates				

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In charge of the PT
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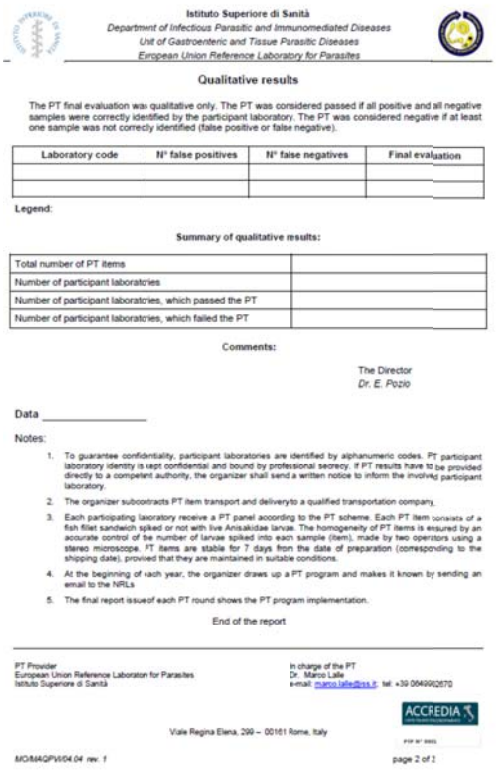

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Qualitative results

The PT final evaluation was qualitative only. The PT was considered passed if all positive and all negative samples were correctly identified by the participant laboratory. The PT was considered negative if at least one sample was not correctly identified (false positive or false negative).

Laboratory code	N° false positives	N° false negatives	Final evaluation

Legend:

Summary of qualitative results:

Total number of PT items	
Number of participant laboratories	
Number of participant laboratories, which passed the PT	
Number of participant laboratories, which failed the PT	

Comments:

The Director
Dr. E. Pozio

Data _____

Notes:

1. To guarantee confidentiality, participant laboratories are identified by alphanumeric codes. PT participant laboratory identity is kept confidential and bound by professional secrecy. If PT results have to be provided directly to a competent authority, the organizer shall send a written notice to inform the involved participant laboratory.
2. The organizer subcontracts PT item transport and delivery to a qualified transportation company.
3. Each participating laboratory receive a PT panel according to the PT scheme. Each PT item consists of a fish fillet sandwich spiked or not with live Anisakidae larvae. The homogeneity of PT items is ensured by an accurate control of the number of larvae spiked into each sample (item), made by two operators using a stereo microscope. PT items are stable for 7 days from the date of preparation (corresponding to the shipping date), provied that they are maintained in suitable conditions.
4. At the beginning of each year, the organizer draws up a PT program and makes it known by sending an email to the NRLs
5. The final report issue of each PT round shows the PT program implementation.

End of the report

PT Provider
European Union Reference Laboratory for Parasites
Istituto Superiore di Sanità

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Annex 4

NRL for parasites	Country
Institut für Veterinärmedizin, Innsbruck	Austria
Institute of Tropical Medicine, Antwerp	Belgium
Cyprus Veterinary Services, Nicosia	Cyprus
State Veterinary Institute, Olomouc	Czech Rep
Technical University of Denmark, National Veterinary Institute, Copenhagen	Denmark
Estonian Veterinary and Food Laboratory, Tartu	Estonia
Finnish Food Safety Authority, Evira, Oulu	Finland
Finnish Food Safety Authority, Evira, Helsinki	Finland
Laboratory for Animal Health, ANSES, Maisons-Alfort	France
Food safety Laboratory, ANSES, Boulogne Sur Mer	France
Max Rubner-Institut, Institut für Sicherheit und Qualität bei Milch und Fisch, Hamburg	Germany
Institute of Infectious and Parasitic Diseases, Athens	Greece
National Food Chain Safety Office, Budapest	Hungary
Veterinary Laboratory Department of Agriculture & Food Laboratories, Celbridge	Ireland
National Reference Laboratory for Anisakiasis, Ist. Zooprof. Sper. Sicilia, Palermo	Italy
Institute of Food Safety, Animal Health and Environment, BIOR, Riga	Latvia
National Food and Veterinary Risk Assessment Institute, Vilnius	Lithuania
National Veterinary Laboratory, Albertown, Marsa	Malta
National Institute of Public Health and the Environment, RIVM, Bilthoven	Netherlands
National Veterinary Research Institute, Pulawy	Poland
Instituto Nacional de Investigacao Agraria e Veterinaria, Oeiras	Portugal
Institute for diagnosis and animal health, Bucharest	Romania
State Veterinary and Food Institute, Bratislava	Slovak Rep.
University of Ljubljana, Veterinary Faculty, Ljubljana	Slovenia
Centro Nacional de Alimentación. Agencia Española de Seguridad Alimentaria y Nutrición, Majadahonda	Spain
Statens Veterinärmedicinska Anstalt, Uppsala	Sweden
Cefas Weymouth Laboratory, Weymouth	UK
Other Laboratories	**Country**
Veterinary Faculty of Sarajevo, Department of Aquaculture, Sarajevo	Bosnia and Herzegovina
Servicio Territorial de Sanidad y Bienestar Social de Salamanca, Salamanca	Spain

Annex 5

Forwarding time and condition of preservation of the package content

Laboratory code	Forwarding days	Time (min/h) between package arrival and opening	Temperature (°C) inside the package at the delivery
A1	1	At arrival	8°C
A2	1	At arrival	6.2°C
A3	1	15 min	4°C
A4	1	At arrival	6°C
A5	1	At arrival	12°C
A6	2	At arrival	4°C
A7	1	At arrival	8°C
A8	1	At arrival	5°C
A10	2	20 min	4°C
A11	2	At arrival	8.8°C
A12	1	1h	6°C
A13	2	At arrival	6°C
A14	1	At arrival	8°C
A15	1	At arrival	3.6°C
A16	2	At arrival	5°C
A18	2	1h15min	5.1°C
A19	1	15 min	10°C
A20	2	15 min	6°C
A21	1	15 min	9°C
A22	2	12h	4°C
A25	1	At arrival	2.3°C
A26	1	At arrival	<10°C
A29	1	15 min	8°C
A30	1	At arrival	7.5°C
A31	1	30min	8.4°C
A32	3	25 min	9°C
A33	1	At arrival	4.3°C

Annex 6

Proficiency test results

Lab code	N° of spiked larvae	N° of detected larvae	N° of spiked larvae	N° of detected larvae	N° of spiked larvae	N° of detected larvae	Method(s)[2]	Final Evaluation
A1	0	0	1	1	3	2	D	positive
A2	0	0	1	1	3	3	D	positive
A3	0	0	1	1	3	2	Co	positive
A4	0	0	1	2 fragments	3	1	D	positive
A5	0	0	1	1	3	3	D	positive
A6	0	0	1	1	3	3	C+D	positive
A7	0	0	1	1	3	3	C+UV+D	positive
A8	0	0	1	1	3	3	D	positive
A10	0	0	1	1	3	3	C	positive
A11	0	1	1	0	3	4 fragments	D	negative
A12	0	0	1	0	3	3	D	negative
A13	0	0	1	1	3	3	D	positive
A14	0	0	1	1	3	3	D	positive
A15	0	0	1	1	3	3	C+UV+D	positive
A16	0	0	1	1	3	3	C+UV	positive
A18	0	0	1	1	3	2	D	positive
A19	0	0	1	1	3	2 + fragments	D	positive
A20	0	0	1	1	3	3	Co+D	positive
A21	0	0	1	1	3	3	D	positive
A22	0	0	1	fragments	3	2 + fragments	D	positive
A25	0	0	1	1	3	2	D	positive
A26	0	0	1	1	3	3	D	positive
A29	0	0	1	1	3	3	UV	positive
A30	0	0	1	1	3	2	C+D	positive
A31	0	0	1	1	3	3	C	positive
A32	0	0	1	1	3	3	D	positive
A33	0	0	1	1	3	3	D	positive

[1]Number of recovered larvae are reported. Number of larval fragments are reported in brackets.
[2]D = digestion; Co = compressorium; C = candling; UV = UV examination after freezing.

Annex 7

Corrective Action results

Lab code	Forwarding days	Temperature in the package at the delivery	N° of spiked larvae	N° of detected larvae	N° of spiked larvae	N° of detected larvae	N° of spiked larvae	N° of detected larvae	Method[1]	Final Evaluation
A11	2	2.5 °C	0	0	1	1	3	2	D	positive
A12	1	cool	0	0	1	1	3	2	D	positive
A17	1	8 °C	0	0	1	1	3	3	UV	positive
A28	1	4.5 °C	0	0	1	1	1 + fragments		UV+D	positive

[1]D = digestion; Co = compressorium; C = candling; UV = UV examination after freezing.